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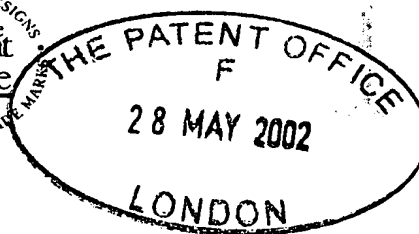
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28 MAY 2002 0212282.8

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P01/7700 0.00-0212282.8

3. Full name, address and postcode of the or of each applicant (*underline all surnames*)

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Patents ADP number (*if you know it*)

08236416002

If the applicant is a corporate body, give the country/state of its incorporation

GB

4. Title of the invention

A METHOD

5. Name of your agent (*if you have one*)

D Young & Co

"Address for service" in the United Kingdom to which all correspondence should be sent (*including the postcode*)

21 New Fetter Lane  
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EC4A 1DA

Patents ADP number (*if you know it*)

59006

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Country

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Signature *D Young & Co* Date 28 May 2002

D Young & Co (Agents for the Applicants)

12. Name and daytime telephone number of person to contact in the United Kingdom Catherine Mallalieu 020 7353 4343

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## A METHOD

### Field of the invention

The present invention relates to a method of detecting modulators of Notch signalling.  
5 The present invention also relates to novel modulators identifiable by such a method and uses thereof in therapy. The present invention also relates to a pharmaceutical composition comprising at least one such modulator.

### Background of the Invention

10

Notch signal transduction plays a critical role in cell fate determination in vertebrate and invertebrate tissues. Notch is expressed at many stages of *Drosophila* embryonic and larval development and in many different cells implying a wide range of functions including an important role in neurogenesis and in the differentiation of mesodermal and  
15 endodermal cells. There are at least four mammalian Notch genes (Notch-1, Notch-2, Notch-3 and Notch-4). Notch-1, which most closely resembles the proteins of invertebrates and lower vertebrates, is widely expressed and is essential for early development. Recent evidence suggests that Notch signalling contributes to lineage commitment of immature T-cells in the thymus.

20

During maturation in the thymus, T-cells acquire the ability to distinguish self-antigens from those that are non-self, a process termed "self tolerance". Tolerance to a non-self antigen, however, may be induced by immunisation under specific conditions with a peptide fragment comprising that antigen. In autoimmune diseases such as multiple  
25 sclerosis, rheumatoid arthritis or diabetes, there is a failure of the proper regulation of tolerance. Improved treatment methods for re-establishing tolerance are desirable for autoimmune diseases. Similarly in allergic conditions and for transplantation of an organ or tissue from a donor individual, induction of tolerance to particular foreign antigens or profiles of foreign antigens is desirable.

30

The expression on the cell surface of normal adult cells of the peripheral immune system of Notch and its ligands, Delta and Serrate, suggests a role for these proteins in T-cell acquired immunocompetence. T-cells express Notch-1 mRNA constitutively. Delta expression is limited to only a subset of T-cells in the peripheral lymphoid tissues. Serrate expression is restricted to a subset of antigen presenting cells (APCs). These observations reinforce the view that the Notch receptor ligand family continues to regulate cell fate decisions in the immune system beyond embryonic development with Notch signalling playing a central role in the induction of peripheral unresponsiveness (tolerance or anergy), linked suppression and infectious tolerance (Hoyne et al.).

10

Thus, as described in WO 98/20142, manipulation of the Notch signalling pathway can be used in immunotherapy and in the prevention and/or treatment of T-cell mediated diseases. In particular, allergy, autoimmunity, graft rejection, tumour induced aberrations to the T-cell system and infectious diseases caused, for example, by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas, Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara, may be targeted.

It has also recently been shown that it is possible to generate a class of regulatory T cells which are able to transmit antigen-specific tolerance to other T cells, a process termed infectious tolerance (WO98/20142). The functional activity of these cells can be mimicked by over-expression of a Notch ligand protein on their cell surfaces or on the surface of antigen presenting cells. In particular, regulatory T cells can be generated by over-expression of a member of the Delta or Serrate family of Notch ligand proteins. Delta or Serrate induced T cells specific to one antigenic epitope are also able to transfer tolerance to T cells recognising other epitopes on the same or related antigens, a phenomenon termed "epitope spreading".

Notch ligand expression also plays a role in cancer. Indeed, upregulated Notch ligand expression has been observed in some tumour cells. These tumour cells are capable of rendering T cells unresponsive to restimulation with a specific antigen, thus providing a possible explanation of how tumour cells prevent normal T cell responses. By

downregulating Notch signalling *in vivo* in T cells, it may be possible to prevent tumour cells from inducing immunotolerance in those T cells that recognise tumour-specific antigens. In turn, this would allow the T cells to mount an immune response against the tumour cells (WO00/135990).

There remains a need in the art for the provision of further diagnostic or therapeutic compositions useful in the detection, prevention and treatment of diseases or conditions of, or relating to, the immune system, and in particular, but not exclusively, T cell mediated diseases or disorders. The present invention addresses this problem by delivering an effective method of identifying novel modulators of the Notch signalling pathway. While many assay methods are known in the art, the present invention is based in our knowledge of the Notch signalling pathway and realisation that an effective assay method for detection of novel modulators needs to be carried out using a cell of the immune system.

5

#### Statements of the Invention

According to one aspect of the present invention there is provided a method for detecting modulators of Notch signalling comprising the step of monitoring Notch  
10 signalling in a cell of the immune system in the presence and absence of a candidate modulator and determining whether the candidate modulator modulates Notch signalling.

According to another aspect of the present invention there is provided a method for  
15 detecting modulators of Notch signalling comprising the steps of:

- (a) contacting a cell of the immune system with a candidate modulator;
- (b) monitoring Notch signalling; and
- (c) determining whether the candidate modulator modulates Notch signalling.

"Contacting" means bringing together in such a way so as the cell may interact with the  
20 candidate modulator. Preferably this will be in an aqueous solvent or buffering solution.

The candidate modulator may be any organic or inorganic compound. Preferably the candidate modulator is selected from a group consisting of: small natural or synthetic molecule compounds, a polypeptide, a polynucleotide, an antibody or a fragment of an antibody and a cytokine or a fragment of a cytokine.

5

In a preferred embodiment, the step of monitoring Notch signalling comprises the steps of monitoring levels of expression of at least one target gene. The target gene may be an endogenous target gene of the Notch signalling pathway or a reporter gene.

- 10 Known endogenous target genes of the Notch signalling pathway include Deltex, Hes-1, E(spl), Il-10, CD-23, Dlx-1, CTLA4, CD-4, Numb, Mastermind and Dsh.

Many reporter genes are standard in the art and include genes encoding an enzymatic activity, genes comprising a radiolabel or a fluorescent label and genes encoding a  
15 predetermined polypeptide epitope.

Preferably at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling. Even more preferably, at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling and  
20 a second signal, and/or a third signal wherein the second and third signals are different.

An example of a signal of use in the present invention is a signal that results from activation of a signalling pathway specific to cells of the immune system, such as a T cell receptor (TCR) signalling pathway, a B cell receptor (BCR) signalling pathway or a  
25 Toll-like receptor (TLR) signalling pathway, with or without an accessory signal (known in the art as costimulatory signals for T and B cell receptor signalling).

Another example of a signal of use in the present invention is a costimulus specific to cells of the immune system such as B7 proteins including B7.1-CD80, B7.2-CD86,  
30 B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD28, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134,

CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205, CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLR) such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FC $\gamma$  receptor 2 (CD32), CD64 (FC $\gamma$  receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors or growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.

In a preferred embodiment, the method of the present invention is carried out in a T cell or T cell progenitor or an antigen presenting cell (APC). APCs are cells which are capable of expressing MHC class II molecules and able to present antigens to CD4<sup>+</sup> T cells. Preferably, the APC will be a myeloid lineage cell such as a dendritic cell, for example a Langerhan cell, a monocyte or macrophage or a primary cell or a B lineage cell.

15

Levels of expression of at least one target gene can be monitored with a protein or a nucleic acid assay.

In accordance with another aspect of the present invention there is provided a method for detecting modulators of Notch signalling comprising the steps of:

- (a) activating a cell of the immune system;
  - (b) contacting the cell with a candidate modulator;
  - (c) monitoring Notch signalling;
- (wherein steps (a), (b) and (c) can be carried out in any order); and
- (d) determining whether the candidate modulator modulates Notch signalling.

Preferably the cell of the immune system is a T-cell.

Preferably the T-cell is activated by activation of the T-cell receptor.

30

Preferably the T-cell receptor is activated with an antigen or antigenic determinant.



Preferably the T-cell receptor is activated by an anti-CD3 antibody

Preferably the T-cell is co-activated

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Preferably the T-cell is co-activated by activation of CD28.

Preferably the T-cell receptor is co-activated by an anti-CD28 antibody.

10 Preferably the anti-CD28 antibody is bound to a support.

Preferably the anti-CD28 antibody is bound to a particulate support.

15 Preferably the T-cell is activated by an anti-CD3 antibody and co-activated by anti-CD28 antibody.

In an preferred embodiment the method comprises the steps of:

- 20 i) activating Notch signalling in the immune cell with a further agent; and  
ii) determining whether the candidate modulator modulates such Notch signalling activation and/or immune cell activation.

Preferably Notch signalling is activated with a Notch ligand.

25 Preferably the Notch ligand is bound to a support.

According to a further aspect of the present invention there is provided a particle comprising a modulator of Notch signalling bound to a particulate support matrix.

30 Preferably the particulate support matrix is a bead.

Preferably the modulator of Notch signalling is a Notch ligand.

Preferably a plurality of Notch ligands are bound to the particulate support matrix.

According to a yet further aspect of the present invention there is provided a modulator identifiable by the method of the invention.

5

According to yet another aspect of the present invention there is provided the use of a modulator according to the present invention in the preparation of a medicament for the treatment of a disease or condition of, or related to the immune system. Preferably, the disease is a T-cell mediated disease.

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According to yet another aspect of the present invention there is provided a pharmaceutical composition comprising a therapeutically effective amount of at least one modulator according to the invention and a pharmaceutically acceptable carrier, diluent and/or excipient.

15

### **Detailed Description**

Various preferred features and embodiments of the present invention will now be described by way of non-limiting examples and with reference to the accompanying  
20 drawings in which:

Figure 1 and Figure 2 show schematic representations of the Notch signalling pathway;

Figure 3 shows a schematic representation of Notch and examples of immune cell  
25 signalling pathways which may be used in screening for immune cell modulators of Notch signalling;

Figures 4 to 6 show schematic representations of the assays of Examples 1 to 9;

Figure 7 shows the results of Example 3;

Figure 8 shows the results of Example 4;

Figure 9 shows the results of Example 5;

Figure 10 shows the results of Example 6;

Figure 11 shows the results of Example 7;

Figure 12 shows the results of Example 8;

5 Figure 13 shows the results of Example 10;

Figures 14-18 show the results of Example 11;

Figure 19 shows the results of Example 12;

Figure 20 shows the results of Example 13;

Figure 21 shows the results of Example 14; and

10 Figures 22A & B, 23, 24A & B and 25 illustrate, and show, the results of Example 15.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general  
25 texts is herein incorporated by reference.

The present invention relates to an assay method for detecting modulators of Notch signalling.

### Notch Signalling

- 5 As used herein, the expression "Notch signalling" is synonymous with the expression "the Notch signalling pathway" and refers to any one or more of the upstream or downstream events that result in, or from, (and including) activation of the Notch receptor.
- 10 Notch signalling directs binary cell fate decisions in the embryo. Notch was first described in *Drosophila* as a transmembrane protein that functions as a receptor for two different ligands, Delta and Serrate. Vertebrates express multiple Notch receptors and ligands. At least four Notch receptors (Notch-1, Notch-2, Notch-3 and Notch-4) have been identified to date in human cells.
- 15 Notch proteins are synthesized as single polypeptide precursors that undergo cleavage via a Furin-like convertase that yields two polypeptide chains that are further processed to form the mature receptor. The Notch receptor present in the plasma membrane comprises a heterodimer of two Notch proteolytic cleavage products, one
- 20 comprising an N-terminal fragment consisting of a portion of the extracellular domain, the transmembrane domain and the intracellular domain, and the other comprising the majority of the extracellular domain. The proteolytic cleavage step of Notch to activate the receptor occurs and is mediated by a furin-like convertase.
- 25 Notch receptors are inserted into the membrane as disulphide-linked heterodimeric molecules consisting of an extracellular domain containing up to 36 epidermal growth factor (EGF)-like repeats and a transmembrane subunit that contains the cytoplasmic domain. The cytoplasmic domain of Notch contains six ankyrin-like repeats, a polyglutamine stretch (OPA) and a PEST sequence. A further domain termed RAM23
- 30 lies proximal to the ankyrin repeats and, like the ankyrin-like repeats, is involved in binding to a transcription factor, known as Suppressor of Hairless [Su(H)] in

*Drosophila* and CBF1 in vertebrates (Tamura). The Notch ligands also display multiple EGF-like repeats in their extracellular domains together with a cysteine-rich DSL (Delta-Serrate Lag2) domain that is characteristic of all Notch ligands (Artavanis-Tsakonas).

5

The Notch receptor is activated by binding of extracellular ligands, such as Delta, Serrate and Scabrous, to the EGF-like repeats of Notch's extracellular domain. Delta requires cleavage for activation. It is cleaved by the ADAM disintegrin metalloprotease Kuzbanian at the cell surface, the cleavage event releasing a soluble and active form of Delta. An oncogenic variant of the human Notch-1 protein, also known as TAN-1, which has a truncated extracellular domain, is constitutively active and has been found to be involved in T-cell lymphoblastic leukemias.

10

The cdc10/ankyrin intracellular-domain repeats mediate physical interaction with intracellular signal transduction proteins. Most notably, the cdc10/ankyrin repeats interact with Suppressor of Hairless [Su(H)]. Su(H) is the *Drosophila* homologue of C-promoter binding factor-1 [CBF-1], a mammalian DNA binding protein involved in the Epstein-Barr virus-induced immortalization of B-cells. It has been demonstrated that, at least in cultured cells, Su(H) associates with the cdc10/ankyrin repeats in the cytoplasm and translocates into the nucleus upon the interaction of the Notch receptor with its ligand Delta on adjacent cells. Su(H) includes responsive elements found in the promoters of several genes and has been found to be a critical downstream protein in the Notch signalling pathway. The involvement of Su(H) in transcription is thought to be modulated by Hairless.

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The intracellular domain of Notch (NotchIC) also has a direct nuclear function (Lieber). Recent studies have indeed shown that Notch activation requires that the six cdc10/ankyrin repeats of the Notch intracellular domain reach the nucleus and participate in transcriptional activation. The site of proteolytic cleavage on the intracellular tail of Notch has been identified between gly1743 and val1744 (termed site 3, or S3)

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(Schroeter). It is thought that the proteolytic cleavage step that releases the NotchIC for nuclear entry is dependent on Presenilin activity.

5 The intracellular domain has been shown to accumulate in the nucleus where it forms a transcriptional activator complex with the CSL family protein CBF1 (suppressor of hairless, Su(H) in *Drosophila*, Lag-2 in *C. elegans*) (Schroeter; Struhl). The NotchIC-CBF1 complexes then activate target genes, such as the bHLH proteins HES (hairy-enhancer of split like) 1 and 5 (Weinmaster). This nuclear function of Notch has also been shown for the mammalian Notch homologue (Lu).

10

NotchIC processing occurs only in response to binding of Notch ligands Delta or Serrate/Jagged. The post-translational modification of the nascent Notch receptor in the Golgi (Munro; Ju) appears, at least in part, to control which of the two types of ligand it interacts with on a cell surface. The Notch receptor is modified on its  
15 extracellular domain by Fringe, a glycosyl transferase enzyme that binds to the Notch/Lin motif. Fringe modifies Notch by adding *O*-linked fucose groups to the EGF-like repeats (Moloney; Bruckner). This modification by Fringe does not prevent ligand binding, but may influence ligand induced conformational changes in Notch. Furthermore, recent studies suggest that the action of Fringe modifies Notch to prevent  
20 it from interacting functionally with Serrate/Jagged ligands but allow it to preferentially interact with Delta (Panin; Hicks). Although *Drosophila* has a single Fringe gene, vertebrates are known to express multiple genes (Radical, Manic and Lunatic Fringes) (Irvine).

25 Thus, signal transduction from the Notch receptor can occur via different pathways (Figures 1-3). The better defined pathway involves proteolytic cleavage of the intracellular domain of Notch (NotchIC) that translocates to the nucleus and forms a transcriptional activator complex with the CSL family protein CBF1 (supressor of hairless, Su(H) in *Drosophila*, Lag-2 in *C. elegans*). NotchIC-CBF1 complexes then  
30 activate target genes, such as the bHLH proteins HES (hairy-enhancer of split like) 1 and 5. Notch can also signal in a CBF1-independent manner that involves the

cytoplasmic zinc finger containing protein Deltex (Figure 3). Unlike CBF1, Deltex does not move to the nucleus following Notch activation but instead can interact with Grb2 and modulate the Ras-Jnk signalling pathway.

- 5 As described above, several endogenous modulators of Notch are already known. These include, for example, the Notch ligands Delta and Serrate. An aim of the present invention is the detection of novel Notch signalling modulators.

#### Candidate Modulators

10

The term "modulate" as used herein refers to a change or alteration in the biological activity of the Notch signalling pathway or a target signalling pathway thereof. The term "modulator" may refer to antagonists or inhibitors of Notch signalling, i.e. compounds which block, at least to some extent, the normal biological activity of the Notch signalling pathway. Conveniently such compounds may be referred to herein as inhibitors or antagonists. Alternatively, the term "modulator" may refer to agonists of Notch signalling, i.e. compounds which stimulate or upregulate, at least to some extent, the normal biological activity of the Notch signalling pathway. Conveniently such compounds may be referred to as upregulators or agonists.

20

The term "candidate modulator" is used to describe any one or more molecule(s) which may be, or is suspected of being, capable of functioning as a modulator of Notch signalling. Said molecules may for example be organic "small molecules" or polypeptides. Suitably, candidate molecules comprise a plurality of, or a library of such molecules or polypeptides. These molecules may be derived from known modulators. "Derived from" means that the candidate modulator molecules preferably comprise polypeptides which have been fully or partially randomised from a starting sequence which is a known modulator of Notch signalling. Most preferably, candidate molecules comprise polypeptides which are at least 40% homologous, more preferably at least 60% homologous, even more preferably at least 75% homologous or even more, for example 85 %, or 90 %, or even more than 95% homologous to one or

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more known Notch modulator molecules, using the BLAST algorithm with the parameters as defined herein.

5 The candidate modulator of the present invention may be an organic compound or other chemical. In this embodiment, the candidate modulator will be an organic compound comprising two or more hydrocarbyl groups. Here, the term "hydrocarbyl group" means a group comprising at least C and H and may optionally comprise one or more other suitable substituents. Examples of such substituents may include halo-, alkoxy-, nitro-, an alkyl group, a cyclic group etc. In addition to the possibility of the  
10 substituents being a cyclic group, a combination of substituents may form a cyclic group. If the hydrocarbyl group comprises more than one C then those carbons need not necessarily be linked to each other. For example, at least two of the carbons may be linked *via* a suitable element or group. Thus, the hydrocarbyl group may contain hetero atoms. Suitable hetero atoms will be apparent to those skilled in the art and  
15 include, for instance, sulphur, nitrogen and oxygen. The candidate modulator may comprise at least one cyclic group. The cyclic group may be a polycyclic group, such as a non-fused polycyclic group. For some applications, the agent comprises at least the one of said cyclic groups linked to another hydrocarbyl group.

20 In one preferred embodiment, the candidate compound will be an amino acid sequence or a chemical derivative thereof, or a combination thereof. In another preferred embodiment, the candidate compound will be a nucleotide sequence, which may be a sense sequence or an anti-sense sequence. The candidate modulator may also be an antibody.

25

Candidate modulators may be synthetic compounds or natural isolated compounds. Various examples of such synthetic or natural modulators are listed below.

**Candidate modulators: antagonists**

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Antagonists of Notch signalling will include any molecule which is capable of inhibiting Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway.

- 5    Candidate modulators for Notch signalling inhibition may be dominant negative versions of a compound capable of activating or transducing Notch signalling. Alternatively, the candidate modulator of Notch signalling will be capable of repressing a compound capable of activating or transducing Notch signalling. In a further alternative embodiment, the modulator will be an inhibitor of Notch signalling.

10

- In a particular embodiment, the modulator will be capable of reducing or preventing Notch or Notch ligand expression. Such a modulator may be a nucleic acid sequence capable of reducing or preventing Notch or Notch ligand expression. Endogenous such modulators include nucleic acid sequences encoding a polypeptide selected from Toll-  
15    like receptor protein family, a cytokine such as IL-12, IFN- $\gamma$ , TNF- $\alpha$ , or a growth factor such as a bone morphogenetic protein (BMP), a BMP receptor and activins. Candidate modulators will include derivatives, fragments, variants, mimetics, analogues and homologues of any of the above.

- 20    In a preferred embodiment, the modulator will be a polypeptide, or a polynucleotide encoding such a polypeptide, that decreases or interferes with the production of compounds that are capable of producing an increase in the expression of Notch ligand. Endogenous compounds of this type include Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors. Candidate modulators will include derivatives, fragments,  
25    variants, mimetics, analogues and homologues of any of the above.

- Alternatively, the candidate modulator will be an antisense construct derived from a sense nucleotide sequence encoding a polypeptide selected from a Notch ligand and a polypeptide capable of up-regulating Notch ligand expression, such as Noggin, Chordin,  
30    Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants, mimetics, analogues and homologues thereof.

In another preferred embodiment the candidate modulator for Notch signalling inhibition will be a molecule which is capable of modulating Notch-Notch ligand interactions. A molecule may be considered to modulate Notch-Notch ligand interactions if it is capable of inhibiting the interaction of Notch with its ligands, preferably to an extent sufficient to provide therapeutic efficacy. In this embodiment the modulator may be a polypeptide, or a polynucleotide encoding such a polypeptide, selected from a Toll-like receptor, a cytokine such as IL-12, IFN- $\gamma$ , TNF- $\alpha$ , or a growth factor such as a BMP, a BMP receptor and activins, derivatives, fragments, variants, mimetics, homologues and analogues thereof. Preferably the modulator will decrease or interfere with the production of an agent that is capable of producing an increase in the expression of Notch ligand, such as Noggin, Chordin, Follistatin, Xnr3, fibroblast growth factors and derivatives, fragments, variants, mimetics homologues and analogues thereof.

Preferably when the modulator is a receptor or a nucleic acid sequence encoding a receptor, the receptor is activated. Thus, for example, when the modulator is a nucleic acid sequence, the receptor is constitutively active when expressed.

Modulators for Notch signalling inhibition also include downstream modulators of the Notch signalling pathway (such as Dsh, Numb and derivatives, fragments, variants, mimetics, homologues and analogues thereof), compounds that prevent expression of Notch target genes or induce expression of genes repressed by the Notch signalling pathway and dominant negative versions of Notch signalling transducer molecules (such as of NotchIC, Deltex and derivatives, fragments, variants, mimetics, homologues and analogues thereof). Proteins for Notch signalling inhibition will also include variants of the wild-type components of the Notch signalling pathway which have been modified in such a way that their presence blocks rather than transduces the signalling pathway. An example of such a modulator would be a Notch receptor which has been modified such that proteolytic cleavage of its intracellular domain is no longer possible.

**Candidate modulators: agonists**

Agonists of Notch signalling will include any molecule which is capable of up-regulating Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway. Candidate modulators for up-regulating the Notch signalling pathway include compounds capable of transducing or activating the Notch signalling pathway.

Modulators for Notch signalling transduction will include molecules which participate in signalling through Notch receptors including activation of Notch, the downstream events of the Notch signalling pathway, transcriptional regulation of downstream target genes and other non-transcriptional downstream events (e.g. post-translational modification of existing proteins). More particularly, such modulators will allow activation of target genes of the Notch signalling pathway.

According to one aspect of the present invention the modulator may be the Notch polypeptide or polynucleotide or a fragment, variant, derivative, mimetic or homologue thereof which retains the signalling transduction ability of Notch or an analogue of Notch which has the signalling transduction ability of Notch. By Notch, we mean Notch-1, Notch-2, Notch-3, Notch-4 and any other Notch homologues or analogues. Analogues of Notch include proteins from the Epstein Barr virus (EBV), such as EBNA2, BARF0 or LMP2A. In a particularly preferred embodiment the modulator may be the Notch intracellular domain (Notch IC) or a sub-fragment, variant, derivative, mimetic, analogue or homologue thereof.

Modulators for Notch signalling activation include molecules which are capable of activating Notch, the Notch signalling pathway or any one or more of the components of the Notch signalling pathway.

Such a modulator may be a dominant negative version of a Notch signalling repressor. In an alternative embodiment, the modulator will be capable of inhibiting a Notch signalling repressor. In a further alternative embodiment, the modulator for Notch signalling activation will be a positive activator of Notch signalling.

In a particular embodiment, the modulator will be capable of inducing or increasing Notch or Notch ligand expression. Such a molecule may be a nucleic acid sequence capable of inducing or increasing Notch or Notch ligand expression.

- 5 In one embodiment, the modulator will be capable of up-regulating expression of the endogenous genes encoding Notch or Notch ligands in target cells. In particular, the modulator may be an immunosuppressive cytokine capable of up-regulating the expression of endogenous Notch or Notch ligands in target cells, or a polynucleotide which encodes such a cytokine. Immunosuppressive cytokines include IL-4, IL-10, IL-10 13, TGF- $\beta$  and FLT3 ligand. Candidate modulators will therefore further include fragments, derivatives, variants, mimetics, analogues and homologues of any of the above.

- Endogenous agonists include Noggin, Chordin, Follistatin, Xnr3, fibroblast growth 15 factors. Candidate modulators may therefore include derivatives, fragments, variants, mimetics, analogues and homologues thereof, or a polynucleotide encoding any one or more of the above.

- In another embodiment, the modulator may be a Notch ligand, or a polynucleotide 20 encoding a Notch ligand. Notch ligands will typically be capable of binding to a Notch receptor polypeptide present in the membrane of a variety of mammalian cells, for example hemapoietic stem cells. Endogenous Notch ligands include polypeptides of the Delta family, for example Delta-1 (Genbank Accession No. AF003522 - *Homo sapiens*), Delta-3 (Genbank Accession No. AF084576 - *Rattus norvegicus*), Delta-like 25 3 (*Mus musculus*), Delta-4 (Genbank Accession No. AB043894) and polypeptides of the Serrate family, for example Serrate-1 and Serrate-2 (WO97/01571, WO96/27610 and WO92/19734), Jagged-1 and Jagged-2 (Genbank Accession No. AF029778 - *Homo sapiens*), and LAG-2. Candidate compounds of the present invention include fragments, derivatives, variants, mimetics, analogues and homologues of any of the 30 above.

In a preferred embodiment, the modulator will be a constitutively active Notch

receptor or Notch intracellular domain, or a polynucleotide encoding such a receptor or intracellular domain.

In an alternative embodiment, the modulator of Notch signalling will act downstream of the Notch receptor. Thus, for example, the activator of Notch signalling may be a constitutively active Deltex polypeptide or a polynucleotide encoding such a polypeptide. Other endogenous downstream components of the Notch signalling pathway include Deltex-1, Deltex-2, Deltex-3, Suppressor of Deltex (SuDx), Numb and isoforms thereof, Numb associated Kinase (NAK), Notchless, Dishevelled (Dsh), emb5, Fringe genes (such as Radical, Lunatic and Manic), PON, LNX, Disabled, Numblake, Nur77, NFkB2, Mirror, Warthog, Engrailed-1 and Engrailed-2, Lip-1 and homologues thereof, the polypeptides involved in the Ras/MAPK cascade modulated by Deltex, polypeptides involved in the proteolytic cleavage of Notch such as Presenilin and polypeptides involved in the transcriptional regulation of Notch target genes. Candidate modulators of use in the present invention will therefore include constitutively active forms of any of the above, analogues, homologues, derivatives, variants, mimetics and fragments thereof.

Modulators for Notch signalling activation may also include any polypeptides expressed as a result of Notch activation and any polypeptides involved in the expression of such polypeptides, or polynucleotides encoding for such polypeptides.

Activation of Notch signalling may also be achieved by repressing inhibitors of the Notch signalling pathway. As such, candidate modulators will include molecules capable of repressing any Notch signalling inhibitors. Preferably the molecule will be a polypeptide, or a polynucleotide encoding such a polypeptide, that decreases or interferes with the production or activity of compounds that are capable of producing an decrease in the expression or activity of Notch, Notch ligands, or any downstream components of the Notch signalling pathway. In a preferred embodiment, the modulators will be capable of repressing polypeptides of the Toll-like receptor protein family, cytokines such as IL-12, IFN- $\gamma$ , TNF- $\alpha$ , and growth factors such as the bone morphogenetic protein (BMP), BMP receptors and activins.

Preferably, the modulator of the present invention will be a polypeptide or a polynucleotide.

- 5 Many compounds identified according to the present invention may be lead compounds useful for drug development. Useful lead compounds include antibodies and peptides, and including intracellular antibodies expressed within the cell in a gene therapy context, which may be used as models for the development of peptide or low molecular weight therapeutics. In a preferred aspect of the invention, lead compounds  
10 and the Notch receptor or Notch ligand or other target peptides may be co-crystallised in order to facilitate the design of suitable low molecular weight compounds which mimic the interaction observed with the lead compound.

#### Polypeptide Sequences

- 15 As used herein, the term "polypeptide" is synonymous with the term "amino acid sequence" and/or the term "protein". In some instances, the term "polypeptide" is synonymous with the term "peptide".

"Peptide" usually refers to a short amino acid sequence that is 10 to 40 amino acids  
20 long, preferably 10 to 35 amino acids.

The polypeptide sequence may be prepared and isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques.

25

#### Polynucleotide Sequences

As used herein, the term "polynucleotide sequence" is synonymous with the term "polynucleotide" and/or the term "nucleotide sequence".

30

The polynucleotide sequence may be DNA or RNA of genomic or synthetic or of recombinant origin. They may also be cloned by standard techniques. The polynucleotide sequence may be double-stranded or single-stranded whether representing the sense or antisense strand or combinations thereof.

5

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length and up to 1,000 bases or even more. Longer polynucleotide sequences will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of  
10 about 15 to 30 nucleotides) flanking a region of the targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction (PCR) under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA.  
15 The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

The nucleic acid may be RNA or DNA and is preferably DNA. Where it is RNA, manipulations may be performed via cDNA intermediates. Generally, a nucleic acid  
20 sequence encoding the first region will be prepared and suitable restriction sites provided at the 5' and/or 3' ends. Conveniently the sequence is manipulated in a standard laboratory vector, such as a plasmid vector based on pBR322 or pUC19 (see below). Reference may be made to Molecular Cloning by Sambrook *et al.* (Cold Spring Harbor, 1989) or similar standard reference books for exact details of the  
25 appropriate techniques.

Sources of nucleic acid may be ascertained by reference to published literature or databanks such as GenBank. Nucleic acid encoding the desired first or second sequences may be obtained from academic or commercial sources where such sources  
30 are willing to provide the material or by synthesising or cloning the appropriate sequence where only the sequence data are available. Generally this may be done by reference to literature sources which describe the cloning of the gene in question.

Alternatively, where limited sequence data is available or where it is desired to express a nucleic acid homologous or otherwise related to a known nucleic acid, exemplary nucleic acids can be characterised as those nucleotide sequences which hybridise to the  
5 nucleic acid sequences known in the art.

The polynucleotide sequence may comprise, for example, a protein-encoding domain, an antisense sequence or a functional motif such as a protein-binding domain and includes variants, derivatives, analogues and fragments thereof. The term also refers to  
10 polypeptides encoded by the nucleotide sequence.

#### Variants, Derivatives, Analogues, Homologues and Fragments

In addition to the specific polypeptide and polynucleotide sequences mentioned herein,  
15 the present invention also encompasses the use of variants, derivatives, analogues, homologues, mimetics and fragments thereof.

In the context of the present invention, a variant of any given sequence is a sequence in which the specific sequence of residues (whether amino acid or nucleic acid residues)  
20 has been modified in such a manner that the polypeptide or polynucleotide in question retains at least one of its endogenous functions. A variant sequence can be modified by addition, deletion, substitution modification replacement and/or variation of at least one residue present in the naturally-occurring protein.

25 The term "derivative" as used herein, in relation to proteins or polypeptides of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of and/or addition of one (or more) amino acid residues from or to the sequence providing that the resultant protein or polypeptide retains at least one of its endogenous functions.

30

The term "analogue" as used herein, in relation to polypeptides or polynucleotides, includes any polypeptide or polynucleotide which retains at least one of the functions



of the endogenous polypeptide or polynucleotide but generally has a different evolutionary origin thereto.

5 The term "mimetic" as used herein, in relation to polypeptides or polynucleotides, refers to a chemical compound that possesses at least one of the endogenous functions of the polypeptide or polynucleotide which it mimics.

10 Typically, amino acid substitutions may be made, for example from 1, 2 or 3 to 10 or 20 substitutions provided that the modified sequence retains the required transport activity or ability to modulate Notch signalling. Amino acid substitutions may include the use of non-naturally occurring analogues.

15 Proteins of use in the present invention may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent protein. Deliberate amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the transport or modulation function is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; 20 and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below.  
25 Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

As used herein, the term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. As used herein, the terms "polypeptide" and "peptide" refer to a polymer in which the monomers are amino acids and are joined together through peptide or disulfide bonds. The terms subunit and domain may also refer to polypeptides and peptides having biological function.

"Fragments" are also variants and the term typically refers to a selected region of the polypeptide or polynucleotide that is of interest either functionally or, for example, in an assay. "Fragment" thus refers to an amino acid or nucleic acid sequence that is a portion of a full-length polypeptide or polynucleotide.

Such variants may be prepared using standard recombinant DNA techniques such as site-directed mutagenesis. Where insertions are to be made, synthetic DNA encoding the insertion together with 5' and 3' flanking regions corresponding to the naturally-occurring sequence either side of the insertion site. The flanking regions will contain convenient restriction sites corresponding to sites in the naturally-occurring sequence so that the sequence may be cut with the appropriate enzyme(s) and the synthetic DNA ligated into the cut. The DNA is then expressed in accordance with the invention to make the encoded protein. These methods are only illustrative of the numerous

standard techniques known in the art for manipulation of DNA sequences and other known techniques may also be used.

Polynucleotide variants will preferably comprise codon optimised sequences. Codon  
5 optimisation is known in the art as a method of enhancing RNA stability and therefore  
gene expression. The redundancy of the genetic code means that several different  
codons may encode the same amino-acid. For example, Leucine, Arginine and Serine  
are each encoded by six different codons. Different organisms show preferences in  
their use of the different codons. Viruses such as HIV, for instance, use a large number  
10 of rare codons. By changing a nucleotide sequence such that rare codons are replaced  
by the corresponding commonly used mammalian codons, increased expression of the  
sequences in mammalian target cells can be achieved. Codon usage tables are known  
in the art for mammalian cells, as well as for a variety of other organisms. Preferably,  
at least part of the sequence is codon optimised. Even more preferably, the sequence is  
15 codon optimised in its entirety.

As used herein, the term "homology" can be equated with "identity". An homologous  
sequence will be taken to include an amino acid sequence which may be at least 75, 85  
or 90% identical, preferably at least 95 or 98% identical. In particular, homology  
20 should typically be considered with respect to those regions of the sequence (such as  
amino acids at positions 51, 56 and 57) known to be essential for an activity.  
Although homology can also be considered in terms of similarity (i.e. amino acid  
residues having similar chemical properties/functions), in the context of the present  
invention it is preferred to express homology in terms of sequence identity.

25

Homology comparisons can be conducted by eye, or more usually, with the aid of  
readily available sequence comparison programs. These commercially available  
computer programs can calculate % homology between two or more sequences.

30 Percent homology may be calculated over contiguous sequences, i.e. one sequence is  
aligned with the other sequence and each amino acid in one sequence is directly  
compared with the corresponding amino acid in the other sequence, one residue at a

time. This is called an “ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximise local homology.

However, these more complex methods assign “gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. “Affine gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

25

Calculation of maximum % homology therefor firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (Devereux). Examples of other software than can perform sequence comparisons include, but are not limited to, the BLAST package, FASTA (Atschul) and the GENEWORKS suite of comparison tools. Both BLAST and FASTA are available for offline and online searching. However it is preferred to use the GCG Bestfit program.

30

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Nucleotide sequences which are homologous to or variants of sequences of use in the present invention can be obtained in a number of ways, for example by probing DNA libraries made from a range of sources. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or part of the reference nucleotide sequence under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the amino acid and/or nucleotide sequences useful in the present invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of use in the present invention. Conserved sequences can be predicted, for example, by aligning the amino

acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used. The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used  
5 for cloning sequences with single sequence primers against known sequences.

Alternatively, such nucleotide sequences may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the  
10 nucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme recognition sites, or to alter the activity of the polynucleotide or encoded polypeptide.

In a first step of the method of the present invention, any one or more of the above  
15 candidate modulators is brought into contact with a cell of the immune system. Cells of the immune system of use in the present invention are described below.

#### Cells of the Immune System

Cells of use in the present invention are cells of the immune system capable of  
20 transducing the Notch signalling pathway.

Most preferably the cells of use in the present invention are T-cells. These include, but are not limited to, CD4<sup>+</sup> and CD8<sup>+</sup> mature T cells, immature T cells of peripheral or thymic origin and NK-T cells.

25 Alternatively, the cells will be antigen-presenting cells (APCs). APCs include dendritic cells (DCs) such as interdigitating DCs or follicular DCs, Langerhans cells, PBMCs, macrophages, B-lymphocytes, T-lymphocytes, or other cell types such as epithelial cells, fibroblasts or endothelial cells, constitutively expressing or activated to  
30 express a MHC Class II molecules on their surfaces. Precursors of APCs include CD34<sup>+</sup> cells, monocytes, fibroblasts and endothelial cells. The APCs or precursors

may be modified by the culture conditions or may be genetically modified, for instance by transfection of one or more genes.

- The T cells or APCs may be isolated from a patient, or from a donor individual or another individual. The cells are preferably mammalian cells such as human or mouse cells. Preferably the cells are of human origin. The APC or precursor APC may be provided by a cell proliferating in culture such as an established cell line or a primary cell culture. Examples include hybridoma cell lines, L-cells and human fibroblasts such as MRC-5. Preferred cell lines for use in the present invention include Jurkat, H9, CEM and EL4 T-cells; long-term T-cell clones such as human HA1.7 or mouse D10 cells; T-cell hybridomas such as DO11.10 cells; macrophage-like cells such as U937 or THP1 cells; B-cell lines such as EBV-transformed cells such as Raji, A20 and M1 cells.
- Dendritic cells (DCs) can be isolated/prepared by a number of means, for example they can either be purified directly from peripheral blood, or generated from CD34<sup>+</sup> precursor cells for example after mobilisation into peripheral blood by treatment with GM-CSF, or directly from bone marrow. From peripheral blood, adherent precursors can be treated with a GM-CSF/IL-4 mixture (Inaba *et al*), or from bone marrow, non-adherent CD34<sup>+</sup> cells can be treated with GM-CSF and TNF- $\alpha$  (Caux *et al*). DCs can also be routinely prepared from the peripheral blood of human volunteers, similarly to the method of Sallusto and Lanzavecchia J Exp Med (1994) 179(4) 1109-18 using purified peripheral blood mononucleocytes (PBMCs) and treating 2 hour adherent cells with GM-CSF and IL-4. If required, these may be depleted of CD19<sup>+</sup> B cells and CD3<sup>+</sup>, CD2<sup>+</sup> T cells using magnetic beads (Coffin *et al*). Culture conditions may include other cytokines such as GM-CSF or IL-4 for the maintenance and, or activity of the dendritic cells or other antigen presenting cells.
- T cells and B cells for use in the invention are preferably obtained from cell lines such as lymphoma or leukemia cell lines, T cell hybridomas or B cell hybridomas but may also be isolated from an individual suffering from a disease of the immune system or a recipient for a transplant operation or from a related or unrelated donor individual. T

cells and B cells may be obtained from blood or another source (such as lymph nodes, spleen, or bone marrow) and may be enriched or purified by standard procedures. Alternatively whole blood may be used or leukocyte enriched blood or purified white blood cells as a source of T cells and other cell types. It is particularly preferred to use  
5 helper T cells (CD4<sup>+</sup>). Alternatively other T cells such as CD8<sup>+</sup> cells may be used.

Candidate modulators of use in the present invention are brought into contact with a cell of the immune system as described above. In a further step, modulation of Notch signalling by a candidate modulator is detected. Assays for detecting modulation of Notch signalling will be described below. Many of these assays will involve  
10 monitoring the expression of a "target gene".

### Target Genes

The target genes of use in the present invention may be endogenous target genes (i.e. endogenous target genes of the Notch signalling pathway) or synthetic reporter genes.  
15

### **Endogenous target genes**

Endogenous target genes of the Notch signalling pathway include Deltex, genes of the Hes family (Hes-1 in particular), Enhancer of Split [E(spl)] complex genes, Il-10, CD-23, Dlx-1, CTLA4, CD-4, Dll-1, Numb, Mastermind and Dsh. Although all genes the  
20 expression of which is modulated by Notch activation may be used for the purpose of the present invention, preferred endogenous target genes are described below.

Deltex, an intracellular docking protein, replaces Su(H) as it leaves its site of interaction with the intracellular tail of Notch, as shown in Figure 1. Deltex is a cytoplasmic protein  
25 containing a zinc-finger (Artavanis-Tsakonas; Osborne). It interacts with the ankyrin repeats of the Notch intracellular domain. Studies indicate that Deltex promotes Notch pathway activation by interacting with Grb2 and modulating the Ras-JNK signalling pathway (Matsuno). Deltex also acts as a docking protein which prevents Su(H) from binding to the intracellular tail of Notch (Matsuno). Thus, Su(H) is released into the



nucleus where it acts as a transcriptional modulator. Recent evidence also suggests that, in a vertebrate B-cell system, Deltex, rather than the Su(H) homologue CBF1, is responsible for inhibiting E47 function (Ordentlich). Expression of Deltex is upregulated as a result of Notch activation in a positive feedback loop. The sequence of Homo sapiens Deltex (DTX1) mRNA may be found in GenBank Accession No. AF053700.

Hes-1 (Hairy-enhancer of Split-1) (Takebayashi) is a transcriptional factor with a basic helix-loop-helix structure. It binds to an important functional site in the CD4 silencer leading to repression of CD4 gene expression. Thus, Hes-1 is strongly involved in the determination of T-cell fate. Other genes from the Hes family include Hes-5 (mammalian Enhancer of Split homologue), the expression of which is also upregulated by Notch activation, and Hes-3. Expression of Hes-1 is upregulated as a result of Notch activation. The sequence of human Hes-1 can be found in GenBank Accession Nos. AK000415 and AF264785.

The E(spl) gene complex [E(spl)-C] (Leimeister) comprises seven genes of which only E(spl) and Groucho show visible phenotypes when mutant. E(spl) was named after its ability to enhance Split mutations, Split being another name for Notch. Indeed, E(spl)-C genes repress Delta through regulation of achaete-scute complex gene expression. Expression of E(spl) is upregulated as a result of Notch activation.

IL-10 (interleukin-10) is a factor produced by Th2 helper T-cells. It is a co-regulator of mast cell growth and shows extensive homology with the Epstein-Barr bcrfi gene. Although it is not known to be a direct downstream target of the Notch signalling pathway, its expression has been found to be strongly upregulated coincident with Notch activation. The mRNA sequence of IL-10 may be found in GenBank ref. No. GI1041812.

CD-23 is the human leukocyte differentiation antigen CD23 (FCE2) which is a key molecule for B-cell activation and growth. It is the low-affinity receptor for IgE. Furthermore, the truncated molecule can be secreted, then functioning as a potent mitogenic growth factor. Although it is not thought to be a direct downstream target of the Notch signalling pathway, its expression has been found to be strongly upregulated coincident with Notch activation. The sequence for CD-23 may be found in GenBank ref. No. GI1783344.

Dlx-1 (distalless-1) expression is downregulated as a result of Notch activation. Sequences for Dlx genes may be found in GenBank Accession Nos. U51000-3.

CTLA4 (cytotoxic T-lymphocyte activated protein 4) is an accessory molecule found on the surface of T-cells which is thought to play a role in the regulation of airway inflammatory cell recruitment and T-helper cell differentiation after allergen inhalation. The promoter region of the gene encoding CTLA4 has CBF1 response elements and its expression is upregulated as a result of Notch activation. The sequence of CTLA4 can be found in GenBank Accession No. L15006.

CD-4 expression is downregulated as a result of Notch activation. A sequence for the CD-4 antigen may be found in GenBank Accession No. XM006966.

### **Synthetic Reporter Genes**

In an alternative embodiment of the present invention, the target gene is a reporter gene. In a preferred embodiment, the reporter gene is under the transcriptional control of a promoter region or responder element(s) sensitive to Notch signalling.

A wide variety of reporters may be used in the assay methods (as well as screens) of the present invention with preferred reporters providing conveniently detectable signals (eg. by spectroscopy). By way of example, a reporter gene may encode an

enzyme which catalyses a reaction which alters light absorption properties.

Other protocols include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA) and fluorescent activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilising monoclonal antibodies reactive to two non-interfering epitopes may even be used. These and other assays are described, among other places, in Hampton R et al (1990, Serological Methods, A Laboratory Manual, APS Press, St Paul MN) and Maddox DE et al.

- 10 One skilled in the art will recognize that the identity of the specific reporter gene can, of course, vary. Examples of reporter genes that have been used in the art include, but are not limited to, genes encoding an enzymatic activity such as chloramphenicol acetyltransferase (CAT) gene, Green Fluorescent Protein (GFP), luciferase (luc),  $\beta$ -galactosidase, invertase, horseradish peroxidase, glucuronidase, exo-glucanase,
- 15 glucoamylase or alkaline phosphatase. Alternatively, the reporter gene may comprise a radiolabel or a fluorescent label such as FITC, rhodamine, lanthanide phosphors, or a green fluorescent fusion protein (See for example Stauber et al). Alternatively, the reporter may comprise a predetermined polypeptide epitope which can be recognized by a secondary reporter such as leucine zipper pair sequences, binding sites for
- 20 secondary antibodies, metal binding domains, or epitope tags. One skilled in the art will appreciate that the specific reporter gene or genes utilized in the methods disclosed herein may vary and may also depend on the specific model system utilized, and the methods disclosed herein are not limited to any specific reporter gene or genes.
- 25 By way of further examples, a number of companies such as Pharmacia Biotech (Piscataway, NJ), Promega (Madison, WI), and US Biochemical Corp (Cleveland, OH) supply commercial kits and protocols for assay procedures. Suitable reporter molecules or labels include those radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors,
- 30 magnetic particles and the like. Patents teaching the use of such labels include US-A-3817837; US-A-3850752; US-A-3939350; US-A-3996345; US-A-4277437; US-A-4275149 and US-A-4366241.

The reporter gene used in the method of the present invention is under the transcriptional control of at least one Notch signalling sensitive promoter region and/or responder element. Promoter regions and/or responder elements sensitive to Notch signalling include the regulatory elements of endogenous Notch target genes such as the HES promoters, Deltex promoter, Notch and Notch ligand promoters, IL-10 promoters. Regulatory elements of use in the present invention also include single or multimerized CBF1 sites, CTLA4 promoters and AIRE promoters. The regulatory elements are positioned such that activation of the Notch signalling pathway results in increased expression of the reporter gene.

One or more copies of the reporter gene can be inserted into the host cell by methods known in the art. The term "host cell" - in relation to the present invention includes any cell that could comprise the target for the agent of the present invention.

Polynucleotides may be introduced into prokaryotic cells or eukaryotic cells, for example yeast, insect or mammalian cells. Preferably, the host cell will be a cell of the immune system as described above.

Polynucleotides of the invention may be introduced into suitable host cells using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

In the present invention, the host cells will preferably be mammalian cells and the polypeptides will be expressed either intracellularly, on the cell membranes or secreted in a culture media if preceded by an appropriate leader sequence.

Expression of the target genes (whether endogenous or synthetic reporter genes) may be dependent on Notch signalling alone or on Notch signalling and one or more further stimulatory signals.

### Stimulatory Signals

5 Expression or repression of the target genes (endogenous or reporter genes) of use in the present invention is dependent on Notch signalling. In a preferred embodiment, expression or repression of the target genes will additionally be depend on a second immune cell specific stimulus, with or without an accessory signal (or "costimulus").

10 In one embodiment, the second stimulus will result from activation of an immune cell receptor. Examples of immune cell receptors include T cell receptors (TCR), B cell receptors (BCR) and Toll-like receptors (TLR). Examples of molecules capable of triggering a TCR or BCR signal include specific antigens for the receptors, superantigens such as TSS1, SEA, SEB, SEC, SED and SEE, antibodies to the TCR  $\alpha\beta$  chains including Fab, F(ab)2 fragments, phage displayed peptides and ScFV or  
15 antibodies to CD3 proteins including  $\xi$  and  $\epsilon$  chains, anti-CD28 antibodies, anti-BCR antibodies, LPS and other bacterial products, cell receptors involved in phagocytosis such as Fc receptors, complement receptors, mannose receptors and other scavenger receptors, receptors involved in clearance of apoptotic cells such as CD36 and  $\alpha v\beta 5$ , dendritic cell receptors such as DEC205 and DC-light, and activators of TCR and/or  
20 BCR signalling pathways such as PMA, ionomycin or kinase inhibitors. These molecules may be used alone or in combination and may be presented on an antigen presenting cell.

25 In accordance with one embodiment of the present invention there is provided a method for detecting modulators of Notch signalling comprising the steps of:

- (a) activating a cell of the immune system;
  - (b) contacting the cell with a candidate modulator;
  - (c) monitoring Notch signalling;
- (wherein steps (a), (b) and (c) can be carried out in any order); and  
30 (d) determining whether the candidate modulator modulates Notch signalling.

Preferably the activator is an anti-CD3 antibody or an anti-CD28 antibody. In more detail, T cell activation involves multiple intracellular signaling events originating from the cell surface TCR/CD3 complex. Cross-linking of the TCR/CD3 complex by anti-CD3 antibodies induces T cell activation, leading to the production of cytokines such as IL-2. IL-2 binds to its high affinity receptor to promote cell proliferation. Additionally co-stimulatory surface molecules such as CD28 have been shown to provide accessory signals in T cell activation, enhancing IL-2 production, e.g. when combined with an anti-CD3 antibody. CD28 is an antigen expressed on the surface of T cells, and is also responsible for activation of T cells.

10

Accessory or costimulatory signals of immune cell receptor signalling include B7 proteins such as B7.1-CD80, B7.2-CD86, B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD28, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134, CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205, CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLRs), such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FC $\gamma$  receptor 2 (CD32), CD64 (FC $\gamma$  receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors and growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.

20

In one embodiment, the second stimulus will be a costimulus. In an alternative embodiment, expression of the target genes will depend on three separate stimuli: Notch signalling, immune cell signalling and a costimulus, all of which are described above. The signals may be delivered all at once or may be phased over a defined period (possibly separated by hours or even days). Preferably, the signals will be delivered substantially simultaneously.

25

### 30 Cell Activation

Immune cell activation may be monitored by any suitable method known to those skilled in the art. For example, cytotoxic activity may be monitored. Natural killer (NK) cells will demonstrate enhanced cytotoxic activity within 4 hours after activation. This cytotoxic activity is maximal after 18 hours.

5

Once activated, leukocytes express a variety of new cell surface antigens. NK cells, for example, will express transferrin receptor, HLA-DR and the CD25 IL-2 receptor after activation. Activation may therefore be assayed by monitoring expression of these antigens.

10

Hara *et al.* Human T Cell Activation: III, Rapid Induction of a Phosphorylated 28 kD/32kD Disulfidelinked Early Activation Antigen (EA-1) by 12-0-tetradecanoyl Phorbol-13-Acetate, Mitogens and Antigens, J. Exp. Med., 164:1988 (1986), and Cosulich *et al.* Functional Characterization of an Antigen (MLR3) Involved in an Early Step of T-Cell Activation, PNAS, 84:4205 (1987), have described cell surface antigens that are expressed on T cells shortly after activation. These antigens, EA-1 and MLR3 respectively, are glycoproteins having major components of 28kD and 32kD. EA-1 and MLR3 are not HLA class II antigens and an MLR3 Mab will block IL-1 binding. These antigens appear on activated T cells within 18 hours and continue to appear as late as 48 hours after activation.

20

These antigens may be useful in detecting leukocyte activation. Additionally, leukocyte activation may be monitored as described in EP O 325 489 which is incorporated herein by reference. Briefly this is accomplished using a monoclonal antibody ("Anti-Leu23") which interacts with a cellular antigen recognised by the monoclonal antibody produced by the hybridoma designated as ATCC No. HB-9627.

25

Anti-Leu 23 recognizes a cell surface antigen on activated and antigen stimulated leukocytes. On activated NK cells, the antigen, Leu 23, is expressed within 4 hours after activation and continues to be expressed as late as 72 hours after activation. Leu 23 is a disulfide-linked homodimer composed of 24 kD subunits with at least two N-linked carbohydrates.

30

Because the appearance of Leu 23 on NK cells correlates with the development of cytotoxicity and because the appearance of Leu 23 on certain T cells correlates with stimulation of the T cell antigen receptor complex, Anti-Leu 23 is useful in monitoring the activation or stimulation of leukocytes.

Further details of techniques for the monitoring of immune cell activation may be found in: 'The Natural Killer Cell' Lewis C.E. and J. O'D. McGee 1992. Oxford University Press; Trinchieri G. 'Biology of Natural Killer Cells' Adv. Immunol. 1989 vol 47 pp187-376; 'Cytokines of the Immune Response' Chapter 7 in "Handbook of Immune Response Genes". Mak T.W. and J.J.L. Simard 1998, which are incorporated herein by reference.

#### Assays

Assays for monitoring expression of the one or more target genes and other methods of detecting modulation of Notch signalling are described below.

The present invention preferably provides a cell-based assay for screening compounds for their ability to modulate Notch signalling. In one embodiment, the present invention provides an assay comprising the steps of:

- (a) providing a culture of immune cells;
- (b) optionally transfecting said cells with a reporter construct;
- (c) optionally transfecting said cells with a Notch gene;
- (d) exposing the cells to one or more compound(s) to be tested; and
- (e) determining the difference in Notch signalling between cells exposed to the compound(s) to be tested and cells not so exposed.

The assay of the present invention is set up to detect either inhibition or enhancement of Notch signalling in cells of the immune system by candidate modulators. The method comprises mixing cells of the immune system, where necessary transformed or transfected, etc. with a synthetic reporter gene, in an appropriate buffer, with a sufficient



amount of candidate modulator and monitoring Notch signalling. The modulators may be small molecules, proteins, antibodies or other ligands as described above. Amounts or activity of the target gene (also described above) will be measured for each compound tested using standard assay techniques and appropriate controls. Preferably the detected  
5 signal is compared with a reference signal and any modulation with respect to the reference signal measured.

The assay may also be run in the presence of a known antagonist of the Notch signalling pathway in order to identify compounds capable of rescuing the Notch signal.

10

Any one or more of appropriate targets - such as an amino acid sequence and/or nucleotide sequence - may be used for identifying a compound capable of modulating the Notch signalling pathway in cells of the immune system in any of a variety of drug screening techniques. The target employed in such a test may be free in solution,  
15 affixed to a solid support, borne on a cell surface, or located intracellularly. The assay of the present invention is a cell based assay.

20

The assay of the present invention may be a screen, whereby a number of agents are tested. In one aspect, the assay method of the present invention is a high through put screen.

25

Techniques for drug screening may be based on the method described in Geysen, European Patent No. 0138855, published on September 13, 1984. In summary, large numbers of different small peptide candidate modulators are synthesized on a solid  
25 substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with a suitable target or fragment thereof and washed. Bound entities are then detected - such as by appropriately adapting methods well known in the art. A purified target can also be coated directly onto plates for use in drug screening techniques. Plates of use for high throughput screening (HTS) will be multi-well plates, preferably  
30 having 96, 384 or over 384 wells/plate. Cells can also be spread as "lawns". Alternatively, non-neutralising antibodies can be used to capture the peptide and

immobilise it on a solid support. High throughput screening, as described above for synthetic compounds, can also be used for identifying organic candidate modulators.

5 This invention also contemplates the use of competitive drug screening assays in which neutralising antibodies capable of binding a target specifically compete with a test compound for binding to a target.

10 It is expected that the assay methods of the present invention will be suitable for both small and large-scale screening of test compounds as well as in quantitative assays.

Various nucleic acid assays are also known. Any conventional technique which is known or which is subsequently disclosed may be employed. Examples of suitable nucleic acid assay are mentioned below and include amplification, PCR, RT-PCR, 15 RNase protection, blotting, spectrometry, reporter gene assays, gene chip arrays and other hybridization methods.

Target gene presence, amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to 20 quantitate the transcription of target mRNA, dot blotting (DNA or RNA analysis), or in situ hybridisation, using an appropriately labelled probe. Those skilled in the art will readily envisage how these methods may be modified, if desired.

25 Generation of nucleic acids for analysis from samples generally requires nucleic acid amplification. Many amplification methods rely on an enzymatic chain reaction (such as a polymerase chain reaction, a ligase chain reaction, or a self-sustained sequence replication) or from the replication of all or part of the vector into which it has been cloned. Preferably, the amplification according to the invention is an exponential amplification, as exhibited by for example the polymerase chain reaction.

Many target and signal amplification methods have been described in the literature, for example, general reviews of these methods in Landegren, U., et al., Science 242:229-237 (1988) and Lewis, R., Genetic Engineering News 10:1, 54-55 (1990). These amplification methods may be used in the methods of our invention, and include  
5 polymerase chain reaction (PCR), PCR in situ, ligase amplification reaction (LAR), ligase hybridisation, Qbeta bacteriophage replicase, transcription-based amplification system (TAS), genomic amplification with transcript sequencing (GAWTS), nucleic acid sequence-based amplification (NASBA) and *in situ* hybridisation. Primers suitable for use in various amplification techniques can be prepared according to  
10 methods known in the art.

PCR is a nucleic acid amplification method described *inter alia* in U.S. Pat. Nos. 4,683,195 and 4,683,202. PCR consists of repeated cycles of DNA polymerase generated primer extension reactions. PCR was originally developed as a means of  
15 amplifying DNA from an impure sample. The technique is based on a temperature cycle which repeatedly heats and cools the reaction solution allowing primers to anneal to target sequences and extension of those primers for the formation of duplicate daughter strands. RT-PCR uses an RNA template for generation of a first strand cDNA with a reverse transcriptase. The cDNA is then amplified according to standard PCR protocol.  
20 Repeated cycles of synthesis and denaturation result in an exponential increase in the number of copies of the target DNA produced. However, as reaction components become limiting, the rate of amplification decreases until a plateau is reached and there is little or no net increase in PCR product. The higher the starting copy number of the nucleic acid target, the sooner this "end-point" is reached. PCR can be used to amplify  
25 any known nucleic acid in a diagnostic context (Mok et al., (1994), Gynaecologic Oncology, 52: 247-252).

Self-sustained sequence replication (3SR) is a variation of TAS, which involves the isothermal amplification of a nucleic acid template via sequential rounds of reverse  
30 transcriptase (RT), polymerase and nuclease activities that are mediated by an enzyme cocktail and appropriate oligonucleotide primers (Guatelli et al. (1990) Proc. Natl. Acad. Sci. USA 87:1874). Enzymatic degradation of the RNA of the RNA/DNA

heteroduplex is used instead of heat denaturation. RNase H and all other enzymes are added to the reaction and all steps occur at the same temperature and without further reagent additions. Following this process, amplifications of  $10^6$  to  $10^9$  have been achieved in one hour at 42 °C.

5

Ligation amplification reaction or ligation amplification system uses DNA ligase and four oligonucleotides, two per target strand. This technique is described by Wu, D. Y. and Wallace, R. B. (1989) *Genomics* 4:560. The oligonucleotides hybridise to adjacent sequences on the target DNA and are joined by the ligase. The reaction is heat  
10 denatured and the cycle repeated.

Alternative amplification technology can be exploited in the present invention. For example, rolling circle amplification (Lizardi *et al.*, (1998) *Nat Genet* 19:225) is an amplification technology available commercially (RCAT™) which is driven by DNA  
15 polymerase and can replicate circular oligonucleotide probes with either linear or geometric kinetics under isothermal conditions.

In the presence of two suitably designed primers, a geometric amplification occurs via DNA strand displacement and hyperbranching to generate  $10^{12}$  or more copies of each  
20 circle in 1 hour.

If a single primer is used, RCAT generates in a few minutes a linear chain of thousands of tandemly linked DNA copies of a target covalently linked to that target.

25 A further technique, strand displacement amplification (SDA; Walker *et al.*, (1992) *PNAS (USA)* 80:392) begins with a specifically defined sequence unique to a specific target. But unlike other techniques which rely on thermal cycling, SDA is an isothermal process that utilises a series of primers, DNA polymerase and a restriction enzyme to exponentially amplify the unique nucleic acid sequence.

30

SDA comprises both a target generation phase and an exponential amplification phase.

In target generation, double-stranded DNA is heat denatured creating two single-stranded copies. A series of specially manufactured primers combine with DNA polymerase (amplification primers for copying the base sequence and bumper primers for displacing the newly created strands) to form altered targets capable of exponential amplification.

The exponential amplification process begins with altered targets (single-stranded partial DNA strands with restricted enzyme recognition sites) from the target generation phase.

An amplification primer is bound to each strand at its complementary DNA sequence. DNA polymerase then uses the primer to identify a location to extend the primer from its 3' end, using the altered target as a template for adding individual nucleotides. The extended primer thus forms a double-stranded DNA segment containing a complete restriction enzyme recognition site at each end.

A restriction enzyme is then bound to the double stranded DNA segment at its recognition site. The restriction enzyme dissociates from the recognition site after having cleaved only one strand of the double-sided segment, forming a nick. DNA polymerase recognises the nick and extends the strand from the site, displacing the previously created strand. The recognition site is thus repeatedly nicked and restored by the restriction enzyme and DNA polymerase with continuous displacement of DNA strands containing the target segment.

25

Each displaced strand is then available to anneal with amplification primers as above. The process continues with repeated nicking, extension and displacement of new DNA strands, resulting in exponential amplification of the original DNA target.

30

In an alternative embodiment, the present invention provides for the detection of gene expression at the RNA level. Typical assay formats utilising ribonucleic acid hybridisation include nuclear run-on assays, RT-PCR and RNase protection assays (Melton *et al.*, Nuc. Acids Res. 12:7035. Methods for detection which can be  
5 employed include radioactive labels, enzyme labels, chemiluminescent labels, fluorescent labels and other suitable labels.

Real-time PCR uses probes labeled with a fluorescent tag or fluorescent dyes and differs from end-point PCR for quantitative assays in that it is used to detect PCR products as  
10 they accumulate rather than for the measurement of product accumulation after a fixed number of cycles. The reactions are characterized by the point in time during cycling when amplification of a target sequence is first detected through a significant increase in fluorescence.

15 The ribonuclease protection (RNase protection) assay is an extremely sensitive technique for the quantitation of specific RNAs in solution. The ribonuclease protection assay can be performed on total cellular RNA or poly(A)-selected mRNA as a target. The sensitivity of the ribonuclease protection assay derives from the use of a complementary *in vitro* transcript probe which is radiolabeled to high specific activity.  
20 The probe and target RNA are hybridized in solution, after which the mixture is diluted and treated with ribonuclease (RNase) to degrade all remaining single-stranded RNA. The hybridized portion of the probe will be protected from digestion and can be visualized via electrophoresis of the mixture on a denaturing polyacrylamide gel followed by autoradiography. Since the protected fragments are analyzed by high  
25 resolution polyacrylamide gel electrophoresis, the ribonuclease protection assay can be employed to accurately map mRNA features. If the probe is hybridized at a molar excess with respect to the target RNA, then the resulting signal will be directly proportional to the amount of complementary RNA in the sample.

30 PCR technology as described e.g. in section 14 of Sambrook *et al.*, 1989, requires the use of oligonucleotide probes that will hybridise to target nucleic acid sequences.

Strategies for selection of oligonucleotides are described below.

As used herein, a probe is e.g. a single-stranded DNA or RNA that has a sequence of nucleotides that includes between 10 and 50, preferably between 15 and 30 and most preferably at least about 20 contiguous bases that are the same as (or the complement of) an equivalent or greater number of contiguous bases. The nucleic acid sequences selected as probes should be of sufficient length and sufficiently unambiguous so that false positive results are minimised. The nucleotide sequences are usually based on conserved or highly homologous nucleotide sequences or regions of polypeptides. The nucleic acids used as probes may be degenerate at one or more positions.

Preferred regions from which to construct probes include 5' and/or 3' coding sequences, sequences predicted to encode ligand binding sites, and the like. For example, either the full-length cDNA clone disclosed herein or fragments thereof can be used as probes. Preferably, nucleic acid probes of the invention are labelled with suitable label means for ready detection upon hybridisation. For example, a suitable label means is a radiolabel. The preferred method of labelling a DNA fragment is by incorporating  $^{32}\text{P}$  dATP with the Klenow fragment of DNA polymerase in a random priming reaction, as is well known in the art. Oligonucleotides are usually end-labelled with  $^{32}\text{P}$ -labelled ATP and polynucleotide kinase. However, other methods (e.g. non-radioactive) may also be used to label the fragment or oligonucleotide, including e.g. enzyme labelling, fluorescent labelling with suitable fluorophores and biotinylation.

Preferred are such sequences, probes which hybridise under high-stringency conditions.

Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature ( $T_m$ ) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is

performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M Na<sup>+</sup> at 65-68 °C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 Na<sup>+</sup> pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, et al., eds. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York or Ausubel, et al., eds. (1990) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.). Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the hybridising pair also play a role.

Gene expression may also be detected using a reporter system. Such a reporter system may comprise a readily identifiable marker under the control of an expression system, e.g. of the gene being monitored. Fluorescent markers, which can be detected and sorted by FACS, are preferred. Especially preferred are GFP and luciferase. Another type of preferred reporter is cell surface markers, i.e. proteins expressed on the cell surface and therefor easily identifiable. Thus, cell-based screening assays can be designed by constructing cell lines in which the expression of a reporter protein, i.e. an easily assayable protein, such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase (CAT) or luciferase, is dependent on the activation of a Notch. For example, a reporter gene encoding one of the above polypeptides may be placed under the control of an response element which is specifically activated by Notch signalling. Alternative assay



formats include assays which directly assess responses in a biological system. If a cell-based assay system is employed, the test compound(s) identified may then be subjected to *in vivo* testing to determine their effect on Notch signalling pathway.

- 5 In general, reporter constructs useful for detecting Notch signalling by expression of a reporter gene may be constructed according to the general teaching of Sambrook et al (1989). Typically, constructs according to the invention comprise a promoter of the gene of interest (i.e. of an endogenous target gene), and a coding sequence encoding the desired reporter constructs, for example of GFP or luciferase. Vectors encoding
- 10 GFP and luciferase are known in the art and available commercially.

Sorting of cells, based upon detection of expression of target genes, may be performed by any technique known in the art, as exemplified above. For example, cells may be sorted by flow cytometry or FACS. For a general reference, see Flow Cytometry and

15 Cell Sorting: A Laboratory Manual (1992) A. Radbruch (Ed.), Springer Laboratory, New York.

Flow cytometry is a powerful method for studying and purifying cells. It has found wide application, particularly in immunology and cell biology: however, the

20 capabilities of the FACS can be applied in many other fields of biology. The acronym F.A.C.S. stands for Fluorescence Activated Cell Sorting, and is used interchangeably with "flow cytometry". The principle of FACS is that individual cells, held in a thin stream of fluid, are passed through one or more laser beams, causing light to be scattered and fluorescent dyes to emit light at various frequencies. Photomultiplier

25 tubes (PMT) convert light to electrical signals, which are interpreted by software to generate data about the cells. Sub-populations of cells with defined characteristics can be identified and automatically sorted from the suspension at very high purity (~100%).

- 30 FACS can be used to measure target gene expression in cells transfected with recombinant DNA encoding polypeptides. This can be achieved directly, by labelling of the protein product, or indirectly by using a reporter gene in the construct.

Examples of reporter genes are  $\beta$ -galactosidase and Green Fluorescent Protein (GFP).  $\beta$ -galactosidase activity can be detected by FACS using fluorogenic substrates such as fluorescein digalactoside (FDG). FDG is introduced into cells by hypotonic shock, and is cleaved by the enzyme to generate a fluorescent product, which is trapped within the cell. One enzyme can therefor generate a large amount of fluorescent product. Cells expressing GFP constructs will fluoresce without the addition of a substrate. Mutants of GFP are available which have different excitation frequencies, but which emit fluorescence in the same channel. In a two-laser FACS machine, it is possible to distinguish cells which are excited by the different lasers and therefor assay two transfections at the same time.

Alternative means of cell sorting may also be employed. For example, the invention comprises the use of nucleic acid probes complementary to mRNA. Such probes can be used to identify cells expressing polypeptides individually, such that they may subsequently be sorted either manually, or using FACS sorting. Nucleic acid probes complementary to mRNA may be prepared according to the teaching set forth above, using the general procedures as described by Sambrook et al (1989).

In a preferred embodiment, the invention comprises the use of an antisense nucleic acid molecule, complementary to a target mRNA, conjugated to a fluorophore which may be used in FACS cell sorting.

Methods have also been described for obtaining information about gene expression and identity using so-called gene chip arrays or high density DNA arrays (Chee). These high density arrays are particularly useful for diagnostic and prognostic purposes. Use may also be made of In Vivo Expression Technology (IVET) (Camilli). IVET identifies target genes up-regulated during say treatment or disease when compared to laboratory culture.

The present invention also provides a method of detection of polypeptides. The advantage of using a protein assay is that Notch activation can be directly measured.

Assay techniques that can be used to determine levels of a polypeptide are well known to those skilled in the art. Such assay methods include radioimmunoassays, competitive-binding assays, protein gel assay, Western Blot analysis, antibody sandwich assays, antibody detection, FACS and ELISA assays. For example,  
5 polypeptides can be detected by differential mobility on protein gels, or by other size analysis techniques, such as mass spectrometry. The detection means may be sequence-specific. For example, polypeptide or RNA molecules can be developed which specifically recognise polypeptides *in vivo* or *in vitro*.

10 For example, RNA aptamers can be produced by SELEX. SELEX is a method for the *in vitro* evolution of nucleic acid molecules with highly specific binding to target molecules. It is described, for example, in U.S. patents 5654151, 5503978, 5567588 and 5270163, as well as PCT publication WO 96/38579

15 The invention, in certain embodiments, includes antibodies specifically recognising and binding to polypeptides.

Antibodies may be recovered from the serum of immunised animals. Monoclonal antibodies may be prepared from cells from immunised animals in the conventional  
20 manner.

The antibodies of the invention are useful for identifying cells expressing the genes being monitored.

25 Antibodies according to the invention may be whole antibodies of natural classes, such as IgE and IgM antibodies, but are preferably IgG antibodies. Moreover, the invention includes antibody fragments, such as Fab, F(ab')<sub>2</sub>, Fv and ScFv. Small fragments, such as Fv and ScFv, possess advantageous properties for diagnostic and therapeutic applications on account of their small size and consequent superior tissue distribution.  
30

The antibodies may comprise a label. Especially preferred are labels which allow the imaging of the antibody in neural cells *in vivo*. Such labels may be radioactive labels

or radioopaque labels, such as metal particles, which are readily visualisable within tissues. Moreover, they may be fluorescent labels or other labels which are visualisable in tissues and which may be used for cell sorting.

- 5 In more detail, antibodies as used herein can be altered antibodies comprising an effector protein such as a label. Especially preferred are labels which allow the imaging of the distribution of the antibody *in vivo*. Such labels can be radioactive labels or radioopaque labels, such as metal particles, which are readily visualisable within the body of a patient. Moreover, they can be fluorescent labels or other labels  
10 which are visualisable on tissue

Antibodies as described herein can be produced in cell culture. Recombinant DNA technology can be used to produce the antibodies according to established procedure, in bacterial or preferably mammalian cell culture. The selected cell culture system  
15 optionally secretes the antibody product, although antibody products can be isolated from non-secreting cells.

Multiplication of hybridoma cells or mammalian host cells *in vitro* is carried out in suitable culture media, which are the customary standard culture media, for example  
20 Dulbecco's Modified Eagle Medium (DMEM) or RPMI 1640 medium, optionally replenished by a mammalian serum, e.g. foetal calf serum, or trace elements and growth sustaining supplements, e.g. feeder cells such as normal mouse peritoneal exudate cells, spleen cells, bone marrow macrophages, 2-aminoethanol, insulin, transferrin, low density lipoprotein, oleic acid, or the like. Multiplication of host cells  
25 which are bacterial cells or yeast cells is likewise carried out in suitable culture media known in the art, for example for bacteria in medium LB, NZCYM, NZYM, NZM, Terrific Broth, SOB, SOC, 2 x YT, or M9 Minimal Medium, and for yeast in medium YPD, YEPD, Minimal Medium, or Complete Minimal Dropout Medium.

- 30 In vitro production provides relatively pure antibody preparations and allows scale-up to give large amounts of the desired antibodies. Techniques for bacterial cell, yeast or mammalian cell cultivation are known in the art and include homogeneous suspension

culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilised or entrapped cell culture, e.g. in hollow fibres, microcapsules, on agarose microbeads or ceramic cartridges.

- 5 Large quantities of the desired antibodies can also be obtained by multiplying mammalian cells in vivo. For this purpose, hybridoma cells producing the desired antibodies are injected into histocompatible mammals to cause growth of antibody-producing tumours. Optionally, the animals are primed with a hydrocarbon, especially mineral oils such as pristane (tetramethyl-pentadecane), prior to the injection. After  
10 one to three weeks, the antibodies are isolated from the body fluids of those mammals. For example, hybridoma cells obtained by fusion of suitable myeloma cells with antibody-producing spleen cells from Balb/c mice, or transfected cells derived from hybridoma cell line Sp2/0 that produce the desired antibodies are injected intraperitoneally into Balb/c mice optionally pre-treated with pristane, and, after one to  
15 two weeks, ascitic fluid is taken from the animals.

The foregoing, and other, techniques are discussed in, for example, Kohler and Milstein, (1975) Nature 256:495-497; US 4,376,110; Harlow and Lane, Antibodies: a Laboratory Manual, (1988) Cold Spring Harbor, incorporated herein by reference.

- 20 Techniques for the preparation of recombinant antibody molecules is described in the above references and also in, for example, EP 0623679; EP 0368684 and EP 0436597, which are incorporated herein by reference.

- The cell culture supernatants are screened for the desired antibodies, preferentially by  
25 an enzyme immunoassay, e.g. a sandwich assay or a dot-assay, or a radioimmunoassay.

- For isolation of the antibodies, the immunoglobulins in the culture supernatants or in the ascitic fluid can be concentrated, e.g. by precipitation with ammonium sulphate,  
30 dialysis against hygroscopic material such as polyethylene glycol, filtration through selective membranes, or the like. If necessary and/or desired, the antibodies are purified by the customary chromatography methods, for example gel filtration, ion-

exchange chromatography, chromatography over DEAE-cellulose and/or (immuno-) affinity chromatography, e.g. affinity chromatography with the target antigen, or with Protein-A.

- 5 The antibody is preferably provided together with means for detecting the antibody, which can be enzymatic, fluorescent, radioisotopic or other means. The antibody and the detection means can be provided for simultaneous, simultaneous separate or sequential use, in a kit.
- 10 The antibodies of the invention are assayed for immunospecific binding by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA, sandwich immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions,
- 15 immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays and protein A immunoassays. Such assays are routine in the art (see, for example, Ausubel et al, eds, 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are
- 20 described briefly below.

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasyolol) supplemented with protein phosphatase and/or protease inhibitors (e. g., EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody of interest to the cell

25 lysate, incubating for a period of time (e. g., 1-4 hours) at 4 °C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4 °C, washing the beads in lysis buffer and resuspending the beads in

30 SDS/sample buffer. The ability of the antibody of interest to immunoprecipitate a particular antigen can be assessed by, e. g., western blot analysis.

- Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (e. g., 8%-20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the
- 5 polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, blocking the membrane in blocking solution (e. g., PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (e. g., PBS-Tween 20), exposing the membrane to a primary antibody (the antibody of interest) diluted in blocking buffer, washing the membrane in washing buffer, exposing the membrane to a secondary antibody (which
- 10 recognises the primary antibody, e. g., an antihuman antibody) conjugated to an enzymatic substrate (e. g., horseradish peroxidase or alkaline phosphatase) or radioactive molecule (e. g.,  $^{32}\text{P}$  or  $^{125}\text{I}$ ) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the antigen.
- 15 ELISAs generally comprise preparing antigen, coating the well of a 96 well microtitre plate with the antigen, adding the antibody of interest conjugated to a detectable compound such as an enzymatic substrate (e. g., horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antigen. In ELISAs the antibody of interest does not have to be
- 20 conjugated to a detectable compound; instead, a second antibody (which recognises the antibody of interest) conjugated to a detectable compound can be added to the well. Further, instead of coating the well with the antigen, the antibody can be coated to the well. In this case, a second antibody conjugated to a detectable compound can be added following the addition of the antigen of interest to the coated well.
- 25
- It is convenient when running assays to immobilise one of more of the reactants, particularly when the reactant is soluble. In the present case it may be convenient to immobilise any one of more of the candidate modulator, Notch ligand, immune cell activator or immune cell costimulus. Immobilisation approaches include covalent
- 30 immobilisation, such as using amine coupling, surface thiol coupling, ligand thiol coupling and aldehyde coupling, and high affinity capture which relies on high affinity

binding of a ligand to an immobilised capturing molecule. Example of capturing molecules include: streptavidin, anti-mouse Ig antibodies, ligand-specific antibodies, protian A, protein G and Tag-specific capture. In one embodiment, immobilisation is achieved through binding to a support, particularly a particulate support which is preferably in the form of a bead.

For assays involving monitoring or detection of tolerised T-cells for use in clinical applications, the assay will generally involve removal of a sample from a patient prior to the step of detecting a signal resulting from cleavage of the intracellular domain.

The invention additionally provides a method of screening for a candidate modulator of Notch signalling, the method comprising mixing in a buffer an appropriate amount of Notch, wherein Notch is suitably labelled with detection means for monitoring cleavage of Notch; and a sample of a candidate ligand; and monitoring any cleavage of Notch.

As used herein, the term "sample" refers to a collection of inorganic, organic or biochemical molecules which is either found in nature (e.g., in a biological- or other specimen) or in an artificially-constructed grouping, such as agents which may be found and/or mixed in a laboratory. The biological sample may refer to a whole organism, but more usually to a subset of its tissues, cells or component parts (e.g. body fluids, including but not limited to blood, mucus, saliva and urine).

The present invention provides a method of detecting novel modulators of Notch signalling. The modulators identified may be used as therapeutic agents – i.e. in therapy applications.

#### TH2 modulation

The humoral/TH2 branch of the immune system is generally directed at protecting



against extracellular immunogens such as bacteria and parasites through the production of antibodies by B cells; whereas the cellular/TH1 branch is generally directed at intracellular immunogens such as viruses and cancers through the activity of natural killer cells, cytotoxic T lymphocytes and activated macrophages (US 6039969). TH2 cells are believed to produce cytokines which stimulate production of IgE antibodies, as well as to be involved with recruitment, proliferation, differentiation, maintenance and survival of eosinophils, which can result in eosinophilia. Eosinophilia is a hallmark of many TH2 mediated diseases, such as asthma, allergy, and atopic dermatitis.

Some diseases that are thought to be caused/mediated in substantial part by TH2 immune response, IL-4/IL-5 cytokine induction, and/or eosinophilia include asthma, allergic rhinitis, systemic lupus erythematosus, Ommen's syndrome (hypereosinophilia syndrome), certain parasitic infections, for example, cutaneous and systemic leishmaniasis, toxoplasma infection and trypanosome infection, and certain fungal infections, for example candidiasis and histoplasmosis, and certain intracellular bacterial infections, such as leprosy and tuberculosis. Additionally, it should also be noted that diseases having a viral or cancer related basis, but with a significant TH2 mediated pathology can also be beneficially treated according to the present invention.

Recent evidence indicates that the immune system can be broken down into two major arms, the humoral and cellular arms. The humoral arm is important in eliminating extracellular pathogens such as bacteria and parasites through production of antibodies by B cells. On the other hand, the cellular arm is important in the elimination of intracellular pathogens such as viruses through the activity of natural killer cells, cytotoxic T lymphocytes and activated macrophages. In recent years it has become apparent that these two arms are activated through distinct T helper cell (TH) populations and their distinct cytokine production profiles. T helper type 1 (TH1) cells are believed to enhance the cellular arm of the immune response and produce predominately the cytokines IL-2 and IFN- $\gamma$ ; whereas, T helper 2 (TH2) cells are believed to enhance the humoral arm of the immune response and produce cytokines, such as interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5) and

granulocyte-macrophage colony-stimulating factor (GM-CSF). In the TH2 case, IL-3, IL-5 and GM-CSF are thought to stimulate eosinophilopoiesis. In addition, IL-5 facilitates terminal differentiation and cell proliferation of eosinophils and promotes survival, viability and migration of eosinophils, while IL-4 stimulates production of antibodies of the IgE class. IgE is an important component in allergies and asthma. IL-5 may also prime eosinophils for the subsequent actions of other mediators.

In contrast, the TH1 cytokines, IL-2 and IFN gamma., are important in activating macrophages, NK cells and CTL (cytotoxic T lymphocytes). IFN gamma also stimulates B cells to secrete specifically cytophilic antibody for the elimination of virally-infected cells. Interestingly, IFN alpha a macrophage-derived cytokine has been shown to antagonize TH2-type responses. IFN alpha also appears to inhibit the proliferation and cytokine production of TH2 cells and enhances IFN gamma production by TH1 cells. In addition, IFN alpha also appears to inhibit IgE production and antigen-induced increases in IL4 mRNA levels.

One common feature of many TH2 mediated diseases is an accumulation of eosinophils, referred to as eosinophilia. For example, chronic pulmonary inflammation involving eosinophil infiltration is a characteristic hallmark feature of bronchial asthma. Increased numbers of eosinophils have been observed in blood, bronchoalveolar lavage fluid and pulmonary tissue in patients with asthma, but the mechanism(s) responsible for their recruitment into and regulation within pulmonary tissues undergoing allergic or pro-inflammatory reactions has not been fully understood. Mediators and cytokines from T-lymphocytes and effector cells such as basophils, mast cells, macrophages and eosinophils have been implicated in enhancing cell maturation, chemotaxis and activation of eosinophils. Evidence suggests that an association exists between the immune system, especially CD4+ T cells, and eosinophils and eosinophil recruitment. Studies in asthmatics and in animal models of allergic pulmonary responses support this notion with the evidence of close correlations between the relative numbers of T cells and activated eosinophils in the airways.

Examples of diseases which may be treated by reducing a TH2 response according to the present invention include include asthma, allergy, atopic dermatitis, early HIV disease, infectious mononucleosis, systemic lupus erythematosus, parasitic infections, for example, cutaneous and systemic leishmaniasis, Toxoplasma infection and  
5 Trypanosome infection, certain fungal infections, for example Candidiasis and Histoplasmosis, and intracellular bacterial infections, such as leprosy and tuberculosis.

#### TNF modulation

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At least two TNFs have been previously described, specifically TNF alpha (TNF alpha) and TNF beta (TNF beta or lymphotoxin), and each is active as a trimeric molecule and is believed to initiate cellular signaling by crosslinking receptors (Engelmann et al. (1990), J. Biol. Chem., 265:14497-14504).

15

Several lines of evidence implicate TNF alpha and TNF beta as major inflammatory cytokines. These known TNFs have important physiological effects on a number of different target cells which are involved in inflammatory responses to a variety of stimuli such as infection and injury. The proteins cause both fibroblasts and synovial  
20 cells to secrete latent collagenase and prostaglandin E2 and cause osteocyte cells to stimulate bone resorption. These proteins increase the surface adhesive properties of endothelial cells for neutrophils. They also cause endothelial cells to secrete coagulant activity and reduce their ability to lyse clots. In addition they redirect the activity of adipocytes away from the storage of lipids by inhibiting expression of the enzyme  
25 lipoprotein lipase. TNFs also cause hepatocytes to synthesize a class of proteins known as "acute phase reactants," which act on the hypothalamus as pyrogens (Selby et al. (1988), Lancet, 1 (8583):483; Starnes, Jr. et al. (1988), J. Clin. Invest., 82:1321; Oliff et al. (1987), Cell, 50:555; and Waage et al. (1987), Lancet, 1 (8529):355).

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Particular examples of diseases which may be treated according to the present invention include, for example:

(A) acute and chronic immune and autoimmune pathologies, such as systemic lupus erythematosus (SLE) rheumatoid arthritis, rheumatoid spondylitis, osteoarthritis, gouty arthritis and other arthritic conditions, thyroidosis, graft versus host disease, scleroderma, diabetes mellitus, Graves'disease, Beschets disease, and the like;

5

(B) infections, including, but not limited to, sepsis syndrome, general sepsis, gram-negative sepsis, septic shock, endotoxic shock, toxic shock syndrome, cachexia, circulatory collapse and shock resulting from acute or chronic bacterial infection, acute and chronic parasitic and/or infectious diseases, bacterial, viral or fungal, such as a HIV, AIDS (including symptoms of cachexia, autoimmune disorders, AIDS dementia complex and infections), fever and myalgias due to bacterial or viral infections;

10

(C) inflammatory diseases, such as chronic inflammatory pathologies and vascular inflammatory pathologies, including chronic inflammatory pathologies such as sarcoidosis, chronic inflammatory bowel disease, ulcerative colitis, and Crohn's pathology and vascular inflammatory pathologies, such as, but not limited to, disseminated intravascular coagulation, atherosclerosis, and Kawasaki's pathology:

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(D) neurodegenerative diseases, including, but are not limited to, demyelinating diseases, such as multiple sclerosis and acute transverse myelitis; extrapyramidal and cerebellar disorders'such as lesions of the corticospinal system; disorders of the basal ganglia or cerebellar disorders; hyperkinetic movement disorders such as Huntington's Chorea and senile chorea; drug-induced movement disorders, such as those induced by drugs which block CNS dopamine receptors; hypokinetic movement disorders, such as Parkinson's disease; Progressive supranucleo palsy; Cerebellar and Spinocerebellar Disorders, such as astructural lesions of the cerebellum; spinocerebellar degenerations (spinal ataxia, Friedreich's ataxia, cerebellar cortical degenerations, multiple systems degenerations (Mencel, Dejerine-Thomas, Shi-Drager, and Machadojoseph)); and systemic disorders (Refsum's disease, abetalipoproteemia, ataxia, telangiectasia, and mitochondrial multi system disorder); demyelinating core disorders, such as multiple sclerosis,

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acute transverse myelitis; disorders of the motor unit, such as neurogenic muscular atrophies (anterior horn cell degeneration, such as amyotrophic lateral sclerosis, infantile spinal muscular atrophy and juvenile spinal muscular atrophy); Alzheimer's disease; Down's Syndrome in middle age; Diffuse Lewy body disease; Senile Dementia of Lewy body type; Wernicke-Korsakoff syndrome; chronic alcoholism; Creutzfeldt-Jakob disease; Subacute sclerosing panencephalitis, Hallerorden-Spatz disease; and Dementia pugilistica, or any subset thereof;

(E) malignant pathologies involving TNF-secreting tumors or other malignancies involving TNF, such as, but not limited to leukemias (acute, chronic myelocytic, chronic lymphocytic and/or myelodysplastic syndrome); lymphomas (Hodgkin's and non-Hodgkin's lymphomas, such as malignant lymphomas (Burkitt's lymphoma or Mycosis fungoides)); carcinomas (such as colon carcinoma) and metastases thereof; cancer-related angiogenesis; infantile haemangiomas;

(F) alcohol-induced hepatitis; and

(G) other diseases related to angiogenesis or VEGF/VPF, such as ocular neovascularization, psoriasis, duodenal ulcers, angiogenesis of the female reproductive tract.

(H) cardiovascular conditions such as atherosclerosis, congestive heart failure, stroke and vasculitis

(I) pulmonary diseases such as adult respiratory distress syndrome (ARDS), chronic pulmonary inflammatory disease, silicosis, asbestosis and pulmonary sarcoidosis.

In one embodiment the present invention may be used to treat a "TNF-mediated disease" A disease or medical condition may be considered to be a " TNF -mediated disease" if the spontaneous or experimental disease is associated with elevated levels

of TNF in bodily fluids or in tissues adjacent to the focus of the disease or indication within the body.

5 Diseases such as rheumatoid arthritis and psoriatic arthritis are chronic joint diseases that afflict and disable, to varying degrees, millions of people worldwide. Rheumatoid arthritis is a disease of articular joints in which the cartilage and bone are slowly eroded away by a proliferative, invasive connective tissue called pannus, which is derived from the synovial membrane. The disease may involve peri-articular structures such as bursae, tendon sheaths and tendons as well as extra-articular tissues such as the  
10 subcutis, cardiovascular system, lungs, spleen, lymph nodes, skeletal muscles, nervous system (central and peripheral) and eyes (Silberberg (1985), Anderson's Pathology, Kissane (ed.), II:1828).

15 There is a wide spectrum of disease severity, but many patients run a course of intermittent relapses and remissions with an overall pattern of slowly progressive joint destruction and deformity. The clinical manifestations may include symmetrical polyarthritis of peripheral joints with-pain, tenderness, swelling and loss of function of affected joints; morning stiffness; and loss of cartilage, erosion of bone matter and subluxation of joints after persistent inflammation. Extra-articular manifestations  
20 include rheumatoid nodules, rheumatoid vasculitis, pleuropulmonary inflammations, scleritis, sicca syndrome, Felty's syndrome (splenomegaly and neutropenia), osteoporosis and weight loss (Katz (1985), Am. J. Med., 79:24 and Krane and Simon (1986), Advances in Rheumatology, Synderman (ed.), 70(2):263-284). The clinical manifestations result in a high degree of morbidity resulting in disturbed daily life of  
25 the patient.

### Therapy

30 The term "therapy" includes curative effects, alleviation effects, and prophylactic effects. The therapy may be on humans or animals.

Modulators identified by the assay method of the present invention may be used to treat disorders and/or conditions of the immune system. In particular, the compounds can be used in the treatment of T cell mediated diseases or disorders. A detailed description of the conditions affected by the Notch signalling pathway may be found in our WO98/20142, WO00/36089 and WO/00135990.

Diseased or infectious states that may be described as being mediated by T cells include, but are not limited to, any one or more of asthma, allergy, tumour induced aberrations to the T cell system and infectious diseases such as those caused by Plasmodium species, Microfilariae, Helminths, Mycobacteria, HIV, Cytomegalovirus, Pseudomonas, Toxoplasma, Echinococcus, Haemophilus influenza type B, measles, Hepatitis C or Toxicara. Thus particular conditions that may be treated or prevented which are mediated by T cells include multiple sclerosis, rheumatoid arthritis and diabetes. The present invention may also be used in organ transplantation or bone marrow transplantation. The present invention is also useful in treating immune disorders such as autoimmune disorders or graft rejection such as allograft rejection.

Examples of autoimmune disorders range from organ specific diseases (such as thyroiditis, insulinitis, multiple sclerosis, iridocyclitis, uveitis, orchitis, hepatitis, Addison's disease, myasthenia gravis) to systemic illnesses such as rheumatoid arthritis or lupus erythematosus. Other disorders include immune hyperreactivity, such as allergic reactions.

In more detail, organ-specific autoimmune diseases include multiple sclerosis, insulin dependent diabetes mellitus, several forms of anemia (aplastic, hemolytic), autoimmune hepatitis, thyroiditis, insulinitis, iridocyclitis, skleritis, uveitis, orchitis, myasthenia gravis, idiopathic thrombocytopenic purpura, inflammatory bowel diseases (Crohn's disease, ulcerative colitis).

Systemic autoimmune diseases include: rheumatoid arthritis, juvenile arthritis, scleroderma and systemic sclerosis, sjogren's syndrom, undifferentiated connective tissue syndrome, antiphospholipid syndrome, different forms of vasculitis

- (polyarteritis nodosa, allergic granulomatosis and angiitis, Wegner's granulomatosis, Kawasaki disease, hypersensitivity vasculitis, Henoch-Schoenlein purpura, Behcet's Syndrome, Takayasu arteritis, Giant cell arteritis, Thrombangiitis obliterans), lupus erythematosus, polymyalgia rheumatica, essentiell (mixed) cryoglobulinemia,
- 5 Psoriasis vulgaris and psoriatic arthritis, diffus fasciitis with or without eosinophilia, polymyositis and other idiopathic inflammatory myopathies, relapsing panniculitis, relapsing polychondritis, lymphomatoid granulomatosis, erythema nodosum, ankylosing spondylitis, Reiter's syndrome, different forms of inflammatory dermatitis.
- 10 A more extensive list of disorders includes: unwanted immune reactions and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease,
- 15 reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic
- 20 diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis,
- 25 intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of degenerative fundus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies,
- 30 acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated



- with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy,
- 5    Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome,
- 10    Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic
- 15    inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery or organ, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g.
- 20    leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.
- 25    The present invention is also useful in cancer therapy, particularly in diseases involving the conversion of epithelial cells to cancer. The present invention is especially useful in relation to adenocarcinomas such as: small cell lung cancer, and cancer of the kidney, uterus, prostate, bladder, ovary, colon and breast.

30    Pharmaceutical Compositions

The present invention provides a pharmaceutical composition comprising

administering a therapeutically effective amount of at least one compound identified by the method of the present invention and a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof).

- 5 The pharmaceutical compositions may be for human or animal usage in human and veterinary medicine and will typically comprise any one or more of a pharmaceutically acceptable diluent, carrier, or excipient. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit.
- 10 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).
- 15 Preservatives, stabilizers, dyes and even flavoring agents may be provided in the pharmaceutical composition. Examples of preservatives include sodium benzoate, sorbic acid and esters of p-hydroxybenzoic acid. Antioxidants and suspending agents may be also used.
- 20 There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestable
- 25 solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.
- 30 Where the compound is to be delivered mucosally through the gastrointestinal mucosa, it should be able to remain stable during transit though the gastrointestinal tract; for example, it should be resistant to proteolytic degradation, stable at acid pH and resistant to the detergent effects of bile.

Where appropriate, the pharmaceutical compositions can be administered by inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

#### 15 Administration

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient. The dosages below are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited.

The compositions of the present invention may be administered by direct injection. The composition may be formulated for parenteral, mucosal, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration.

25 The term "administered" includes delivery by viral or non-viral techniques. Viral delivery mechanisms include but are not limited to adenoviral vectors, adeno-associated viral (AAV) vectors, herpes viral vectors, retroviral vectors, lentiviral vectors, and baculoviral vectors. Non-viral delivery mechanisms include lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. The routes for such delivery mechanisms include but are not limited to mucosal, nasal, oral, parenteral,

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gastrointestinal, topical, or sublingual routes.

The term "administered" includes but is not limited to delivery by a mucosal route, for example, as a nasal spray or aerosol for inhalation or as an ingestible solution; a  
5 parenteral route where delivery is by an injectable form, such as, for example, an intravenous, intramuscular, intradermal, intra-articular, intrathecal, intra-peritoneal or subcutaneous route, or via the alimentary tract (for example, via the Peyers patches).

The routes of administration and dosages described are intended only as a guide since  
10 a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient depending on, for example, the age, weight and condition of the patient. Preferably the pharmaceutical compositions are in unit dosage form. The present invention includes both human and veterinary applications.

15

The present invention will now further be described with reference to the following non-limiting Examples:

### Example 1

20

#### **CD4+ cell purification**

Spleens were removed from mice (variously Balb/c females, 8-10 weeks, C57B/6 females, 8-10 weeks, CARD1 females, 8-10 weeks (D011.10 transgenic, CAR transgenic)) and passed through a 0.2 $\mu$ M cell strainer into 20ml R10F medium (R10F-  
25 RPMI 1640 media (Gibco Cat No 22409) plus 2mM L-glutamine, 50 $\mu$ g/ml Penicillin, 50 $\mu$ g/ml Streptomycin, 5 x 10<sup>-5</sup> M  $\beta$ -mercapto-ethanol in 10% fetal calf serum). The cell suspension was spun (1150rpm 5min) and the media removed.

The cells were incubated for 4 minutes with 5ml ACK lysis buffer (0.15M NH<sub>4</sub>Cl,  
30 1.0M KHCO<sub>3</sub>, 0.1mM Na<sub>2</sub>EDTA in double distilled water) per spleen (to lyse red blood cells). The cells were then washed once with R10F medium and counted. CD4+

cells were purified from the suspensions by positive selection on a Magnetic Associated Cell Sorter (MACS) column (Miltenyi Biotec, Bisley, UK: Cat No 130-042-401) using CD4 (L3T4) beads (Miltenyi Biotec Cat No 130-049-201), according to the manufacturer's directions.

5

### **Example 2**

#### **Antibody Coating**

The following protocols were used for coating 96 well flat-bottomed plates with antibodies.

10

A) The plates were coated with Dulbecco's Phosphate Buffered Saline (DPBS) plus 1µg/ml anti-CD3 antibody (Pharmingen, San Diego, US: Cat No 553058, Clone No 145-2C11) plus 1µg/ml anti-IgG4 antibody (Pharmingen Cat No 555878). 100µl of coating mixture was used per well. Plates were incubated overnight at 4°C then washed with DPBS. Each well then received either 100µl DPBS or 100µl DPBS plus 10µg/ml Notch ligand (mouse Delta 1 extracellular domain/Ig4Fc fusion protein; Fc-delta).

15

The plates were incubated for 2-3 hours at 37°C then washed again with DPBS before cells (prepared as in Example 1) were added.

20

B) Alternatively, the plates were coated with DPBS plus 1µg/ml anti-hamsterIgG antibody (Pharmingen Cat No 554007) plus 1µg/ml anti-IgG4 antibody. 100µl of coating mixture was added per well. Plates were incubated overnight at 4°C then washed with DPBS. Each well then received either 100µl DPBS plus anti-CD3 antibody (1µg/ml) or, 100µl DPBS plus anti-CD3 antibody (1µg/ml) plus Fc-delta (10µg/ml). The plates were incubated for 2-3 hours at 37°C then washed again with DPBS before cells (prepared as in Example 1) were added.

25

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**Example 3****Primary Polyclonal Stimulation**

CD4<sup>+</sup> cells were cultured in 96 well, flat-bottomed plates pre-coated according to  
5 Example 2 (A) or 2 (B). Cells were re-suspended, following counting, at  $2 \times 10^6$  /ml in  
R10F medium plus 4µg/ml anti-CD28 antibody (Pharmingen, Cat No 553294, Clone  
No 37.51). 100µl cell suspension was added per well. 100µl of R10F medium was  
then added to each well to give a final volume of 200µl ( $2 \times 10^5$  cells/well, anti-CD28  
final concentration 2µg/ml) The plates were then incubated at 37°C for 72 hours.  
10  
125µl supernatant was then removed from each well and stored at -20°C until tested by  
ELISA for IL-10, IFNγ and IL-13 using antibody pairs from R & D Systems  
(Abingdon, UK). The cells were then split 1 in 3 into new wells (not coated) and fed  
with R10F medium plus recombinant human IL-2 (2.5ng/ml, PeproTech Inc, London,  
15 UK: Cat No 200-02).

Results are shown in Figure 7.

**Example 4**

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**Real Time PCR analysis of primary stimulated CD4<sup>+</sup> cells**

Murine (Balb/c) stimulated CD4<sup>+</sup> T-cells from Example 3 were harvested at 4, 16 and  
24 hours. Total cellular RNA was isolated using the RNeasy<sup>TM</sup> RNA isolation kit  
(Qiagen, Crawley, UK) according to the manufacturer's guidelines.

25

In each case 1µg of total RNA was reverse transcribed using SuperScript<sup>TM</sup> II Reverse  
Transcriptase (Invitrogen, Paisley, UK) using Oligo dT<sub>(12-18)</sub> or a random decamer mix  
according to the manufacturer's guidelines. After synthesis, Oligo dT<sub>(12-18)</sub>- and  
random decamer-primed cDNAs were mixed in equal proportions to provide the  
30 working cDNA sample for real-time quantitative PCR analysis.

Real-time quantitative PCR was performed using the Roche Lightcycler<sup>TM</sup> system (Roche, UK) and SYBR green detection chemistry according to the manufacturer's guidelines. The following HPLC-purified primer pairs were used for cDNA-specific amplification (5' to 3'):

5

*mouse 18s rRNA:*      **Forward** GTAACCCGTTGAACCCCAT  
                                 **Reverse** CCATCCAATCGGTAGTAGCG

10

*mouse Hes-1:*            **Forward** GGTGCTGATAACAGCGGAAT  
                                 **Reverse** ATTTTGGGAATCCTTCACGC

The endpoint used in real-time PCR quantification, the Crossing Point ( $C_p$ ), is defined as the PCR cycle number that crosses an algorithm-defined signal threshold.

Quantitative analysis of gene-specific cDNA was achieved firstly by generating a set of standards using the  $C_p$ s from a set of serially-diluted gene-specific amplicons which had been previously cloned into a plasmid vector (pCR2.1, Invitrogen). These serial dilutions fall into a standard curve against which the  $C_p$ s from the cDNA samples were compared. Using this system, expression levels of the 18S rRNA house-keeping gene were generated for each cDNA sample. Hes-1 was then analysed by the same method using serially-diluted Hes-1-specific standards, and the Hes-1 value divided by the 18S rRNA value to generate a value, which represents the relative expression of Hes-1 in each cDNA sample. All  $C_p$  analysis was performed using the Second Derivative Maximum algorithm within the Lightcycler system software.

Results (HES-1 expression relative to 18S rRNA expression with and without Fc-delta) are shown in Figure 8.

### Example 5

#### 30    **Screening under polarising conditions**

Plates were coated and CD4<sup>+</sup> cells added as in Example 2 (A).

The procedure of Example 3 was then followed, except that instead of adding 100 $\mu$ l R10F medium per well as in Example 3, 100 $\mu$ l of polarising cocktail was added per well as follows:

- 5    Un-polarised cells: R10F medium.  
     Th1 polarised cells: R10F medium plus anti-IL-4 antibody (10 $\mu$ g/ml, Pharmingen Cat No 554432) plus IL-12 (10ng/ml, Peprotech 210-12).  
     Th2 polarised cells: R10Fmedium plus anti-IL-12 antibody (10 $\mu$ g/ml, Pharmingen Cat No 554475) plus anti-IFN $\gamma$  antibody (1 $\mu$ g/ml, Pharmingen Cat No 554408) plus IL-4  
10    (10ng/ml, Peprotech Cat No 214-14).

Cells were then stimulated and cytokines (IL-10, IFN $\gamma$  and IL-13) measured by ELISA as described in Example 3. Results are shown in Figure 9.

15    **Example 6**

**Soluble Ligand**

- The procedure of Example 2(A) (with the modification that ligand was not added to the plate) and Example 3 (with the modification that soluble Fc-delta was added with  
20    the R10F medium) was used to compare soluble Fc-delta with plate-bound Fc-delta against controls. Results are shown in Figure 10.

**Example 7**

25    **Secondary stimulation**

7 days after primary stimulation all cells were harvested and counted then stimulated in one of three ways as follows:

**Re-stimulation**

- 30    Cells were re-stimulated exactly as for primary stimulation (Example 3).



**Re-challenge on anti-CD3/CD28**

96-well flat-bottomed plates were coated with PBS plus 1 µg/ml anti-CD3 antibody. The plates were incubated overnight at 4°C then washed with DPBS.

- 5 The cells were re-suspended at  $2 \times 10^6$  /ml in R10F medium plus anti-CD28 antibody (4 µg/ml). 100 µl cell suspension was added per well. 100 µl of R10F medium was then added per well to give a final volume of 200 µl. ( $2 \times 10^5$  cells/well, anti-CD28 final concentration 2 µg/ml). The plates were then incubated at 37°C for 72 hours. After 72 hours supernatants were removed for ELISA as described in Example 3 (primary  
10 stimulation).

**Re-stimulation with APC plus anti-CD3**

Primary stimulated cells from Example 3 were harvested after 7 days and restimulated with APCs of the same strain ( $2 \times 10^4$  per well) plus anti-CD3 antibody.

- 15 Mouse spleen cells were isolated as described in Example 1 up to the counting step. Thy-1.2 antibody-binding cells were then removed on a MACS column and the flowthrough was recovered and treated with mitomycin-C for 45 minutes then added to a 96 well plate in 100 µl R10F medium with equal numbers of cells from Example 3  
20 and 0.5 µg/ml anti-CD3 antibody.

- Cell proliferation was measured using a kit from Roche Molecular Biochemicals, Cell Proliferation ELISA, BrdU (chemiluminescent) 1 669 915, according to the manufacturer's instructions. Plates were pulsed at 72 hours and read on a luminometer.  
25

Cytokines (IL-10 and IFN-γ) were measured as described in Example 3. Results are shown in Figure 11.

**Example 8****CHO-N2 (N27) Luciferase Reporter Assay****5    A) Construction of Luciferase Reporter Plasmid 10xCBF1-Luc (pLOR91)**

An adenovirus major late promoter TATA-box motif with BglII and HindIII cohesive ends was generated as follows:

10    **BglII** **HindIII**

GATCTGGGGGGCTATAAAAGGGGGTA  
           ACCCCCGATATTTCCCCCATTCTGA

This was cloned into plasmid pGL3-Basic (Promega) between the BglII and HindIII  
 15    sites to generate plasmid pGL3-AdTATA.

A TP1 promoter sequence (TP1; equivalent to 2 CBF1 repeats) with BamH1 and BglII  
 cohesive ends was generated as follows:

20    BamH1 BglII  
 5' GATCCCGACTCGTGGGAAAATGGGCGGAAGGGCACCGTGGGAAAATAGTA 3'  
       3'            GGCTGAGCACCTTTTACCCGCCTTCCCGTGGCACCTTTTATCATCTAG 5'

This sequence was pentamerised by repeated insertion into a BglII site and the  
 25    resulting TP1 pentamer (equivalent to 10 CBF1 repeats) was inserted into pGL3-  
 AdTATA at the BglII site to generate plasmid pLOR91.

**B) Generation of a stable CHO cell reporter cell line expressing full length Notch2  
 and the 10xCBF1-Luc reporter cassette**

30

A cDNA clone spanning the complete coding sequence of the human Notch2 gene  
 (see, eg GenBank Accession No AF315356) was constructed as follows. A 3' cDNA

fragment encoding the entire intracellular domain and a portion of the extracellular domain was isolated from a human placental cDNA library (OriGene Technologies Ltd., USA) using a PCR-based screening strategy. The remaining 5' coding sequence was isolated using a RACE (Rapid Amplification of cDNA Ends) strategy and ligated  
5 onto the existing 3' fragment using a unique restriction site common to both fragments (Cla I). The resulting full-length cDNA was then cloned into the mammalian expression vector pcDNA3.1-V5-HisA (Invitrogen) without a stop codon to generate plasmid pLOR92. When expressed in mammalian cells, pLOR92 thus expresses the full-length human Notch2 protein with V5 and His tags at the 3' end of the intracellular  
10 domain.

Wild-type CHO-K1 cells (eg see ATCC No CCL 61) were transfected with pLOR92 (pcDNA3.1-FLNotch2-V5-His) using Lipfectamine 2000<sup>TM</sup> (Invitrogen) to generate a stable CHO cell clone expressing full length human Notch2 (N2). Transfectant clones  
15 were selected in Dulbecco's Modified Eagle Medium (DMEM) plus 10% heat inactivated fetal calf serum ((HI)FCS) plus glutamine plus Penicillin-Streptomycin (P/S) plus 1 mg/ml G418 (Geneticin<sup>TM</sup> – Invitrogen) in 96-well plates using limiting dilution. Individual colonies were expanded in DMEM plus 10%(HI)FCS plus glutamine plus P/S plus 0.5 mg/ml G418. Clones were tested for expression of N2 by  
20 Western blots of cell lysates using an anti-V5 monoclonal antibody (Invitrogen). Positive clones were then tested by transient transfection with the reporter vector pLOR91 (10xCBF1-Luc) and co-culture with a stable CHO cell clone (CHO-Delta) expressing full length human delta-like ligand 1 (DLL1; eg see GenBank Accession No AF196571). (CHO-Delta was prepared in the same way as the CHO Notch 2 clone,  
25 but with human DLL1 used in place of Notch 2. A strongly positive clone was selected by Western blots of cell lysates with anti-V5 mAb.)

One CHO-N2 stable clone, N27, was found to give high levels of induction when transiently transfected with pLOR91 (10xCBF1-Luc) and co-cultured with the stable  
30 CHO cell clone expressing full length human DLL1 (CHO-Delta1). A hygromycin gene cassette (obtainable from pcDNA3.1/hygro, Invitrogen) was inserted into pLOR91 (10xCBF1-Luc) using BamH1 and Sal1 and this vector (10xCBF1-Luc-

hygro) was transfected into the CHO-N2 stable clone (N27) using Lipfectamine 2000 (Invitrogen). Transfectant clones were selected in DMEM plus 10%(HI)FCS plus glutamine plus P/S plus 0.4 mg/ml hygromycin B (Invitrogen) plus 0.5 mg/ml G418 (Invitrogen) in 96-well plates using limiting dilution. Individual colonies were  
5 expanded in DMEM plus 10%(HI)FCS plus glutamine plus P/S + 0.2 mg/ml hygromycin B plus 0.5 mg/ml G418 (Invitrogen).

Clones were tested by co-culture with a stable CHO cell clone expressing FL human DLL1. Three stable reporter cell lines were produced N27#11, N27#17 and N27#36.  
10 N27#11 was selected for further use because of its low background signal in the absence of Notch signalling, and hence high fold induction when signalling is initiated. Assays were set up in 96-well plates with  $2 \times 10^4$  N27#11 cells per well in 100  $\mu$ l per well of DMEM plus 10%(HI)FCS plus glutamine plus P/S.

15 **C) Transient Transfection of CHO-N2 Cells with 10xCBF1-Luc**

Alternatively, for transient transfection, CHO-N2 (Clone N27) cells were maintained in DMEM plus 10%(HI)FCS plus glutamine plus P/S plus 0.5 mg/ml G418 and a T<sub>80</sub> flask of the CHO-N2 cells was transfected as follows. The medium on the cells was  
20 replaced with 8 ml of fresh in DMEM plus 10%(HI)FCS plus glutamine plus P/S. In a sterile bijou 10  $\mu$ g of pLOR91 (10xCBF1-Luc) was added to OptiMem (Invitrogen) to give a final volume of 1 ml and mixed. In a second sterile bijou 20  $\mu$ l of Lipofectamine 2000 reagent was added to 980  $\mu$ l of OptiMem and mixed.

25 The contents of each bijou were mixed and left at room temperature for 20 minutes. The 2 ml of transfection mixture was added to the flask of cells containing 8 ml of medium and the resulting mixture was left in a CO<sub>2</sub> incubator overnight before removing the transfected cells and adding to the 96-well plate containing the immobilised Notch ligand protein.

30

The following day the transfected CHO-N2 cells were removed using 0.02% EDTA solution (Sigma), spun down and resuspended in 10 ml DMEM plus 10%(HI)FCS plus

- glutamine plus P/S. 10  $\mu$ l of cells were counted and the cell density was adjusted to  $2.0 \times 10^5$  cells/ml with fresh DMEM plus 10%(HI)FCS plus glutamine plus P/S. 100  $\mu$ l per well was added to a 96-well tissue culture plate (flat bottom), i.e.  $2.0 \times 10^4$  transfected cells per well, using a multi-channel pipette and the plate was then
- 5 incubated overnight.

**D) Immobilisation of Notch Ligand protein directly onto a 96-well Tissue Culture Plate**

- 10 10  $\mu$ g of purified Notch ligand protein was added to sterile PBS in a sterile Eppendorf tube to give a final volume of 1 ml. Serial 1:2 dilutions were made by adding 500  $\mu$ l into sterile Eppendorf tubes containing 500  $\mu$ l of sterile PBS to generate dilutions of 10  $\mu$ g/ml, 5  $\mu$ g/ml, 2.5  $\mu$ g/ml, 1.25  $\mu$ g/ml, 0.625  $\mu$ g/ml and 0  $\mu$ g/ml.
- 15 The lid of the plate was sealed with parafilm and the plate was left at 4 °C overnight or at 37 °C for 2 hours. The protein was then removed and the plate was washed with 200  $\mu$ l of PBS.

**E) A20-Delta cells**

- 20 The IVS, IRES, Neo and pA elements were removed from plasmid pIRESneo2 (Clontech, USA) and inserted into a pUC cloning vector downstream of a chicken beta-actin promoter (eg see GenBank Accession No E02199). Mouse Delta-1 (eg see GenBank Accession No NM\_007865) was inserted between the actin promoter and
- 25 IVS elements and a sequence with multiple stop codons in all three reading frames was inserted between the Delta and IVS elements.

The resulting construct was transfected into A20 cells using electroporation and G418 to provide A20 cells expressing mouse Delta1 on their surfaces (A20-Delta).

**F) CHO and CHO-hDelta1-V5-His Assay Control**

CHO cells were maintained in DMEM plus 10%(HI)FCS plus glutamine plus P/S and CHO-hDelta1-V5-His (clone#10) cells were maintained in DMEM plus 10%(HI)FCS plus glutamine plus P/S plus 0.5mg/ml G418.

Cells were removed using 0.02% EDTA solution (Sigma), spun down and resuspended in 10 ml DMEM plus 10%(HI)FCS plus glutamine plus P/S. 10 µl of cells were counted and the cell density was adjusted to  $5.0 \times 10^5$  cells/ml with fresh DMEM plus 10%(HI)FCS plus glutamine plus P/S. 300 µl of each cell line at  $5.0 \times 10^5$  cells/ml was added into duplicate wells of a 96-well tissue culture plate. 150 µl of DMEM plus 10%(HI)FCS plus glutamine plus P/S was added in to the next 5 wells below each well. 150 µl of cells were serially diluted into the next 4 wells giving cell density dilution of  $5.0 \times 10^5$  cells/ml,  $2.5 \times 10^5$  cells/ml,  $1.25 \times 10^5$  cells/ml,  $0.625 \times 10^5$  cells/ml,  $0.3125 \times 10^5$  cells/ml and 0 cells/ml.

100 µl from each well was added into the 96-well plate containing 100 µl of CHO-N2 cells transfected with 10xCBF1-Luc ( $2.0 \times 10^4$  transfected CHO-N2 cells/well) and the plate was left in an incubator overnight.

**G) Cell Co-Culture**

$5 \times 10^4$  CHO-N2 cells were plated on a 96 well plate. CHO-Delta or A20-Delta cells were titrated in as required (max ratio CHO-N2: CHO-Delta was 1:1, max ratio CHO-N2: A20-Delta was 1:2). The mixture was incubated overnight before conducting a luciferase assay.

**H) Luciferase Assay**

Supernatant was removed from all wells. 100 µl of PBS and 100 µl of SteadyGlo™ luciferase assay reagent (Promega) was added and the cells were left at room temperature for 5 minutes. The mixture was pipetted up and down 2 times to ensure

cell lysis and contents from each well were transferred into a white 96-well OptiPlate™ (Packard). Luminescence was measured in a TopCount™ counter (Packard).

- 5 Results of sample assays (using the stable CHO-Notch2-10xCBF1-Luc reporter cell line described above with (A) plate-immobilised human Delta-1/Ig4Fc fusion protein, (B) plate-immobilised mouse Delta-1/Ig4Fc fusion protein, (C) CHO / CHO-human Delta1 co-cultured cells and (D) A20/ A20-mouse Delta1 co-cultured cells as actives against corresponding controls) are shown in Figures 12 A to D.

10

### **Example 9**

#### **Dynabeads Luciferase Assay Method For Detecting Notch Ligand Activity**

- 15 Fc-tagged Notch ligands were immobilised on Streptavidin-Dynabeads (CELLlection Biotin Binder Dynabeads [Cat. No. 115.21] at  $4.0 \times 10^8$  beads/ml from Dynal (UK) Ltd; beads) in combination with biotinylated  $\alpha$ -IgG-4 (clone JDC14 at 0.5 mg/ml from Pharmingen [Cat. No. 555879]) as follows:

- 20  $2.5 \times 10^7$  beads (62.5  $\mu$ l of beads at  $4.0 \times 10^8$  beads/ml) and 5  $\mu$ g biotinylated  $\alpha$ -IgG-4 was used for each sample assayed. PBS was added to the beads to 1 ml and the mixture was spun down at 13,000 rpm for 1 minute. Following washing with a further 1 ml of PBS the mixture was spun down again. The beads were then resuspended in a final volume of 100  $\mu$ l of PBS containing the biotinylated  $\alpha$ -IgG-4 in a sterile Eppendorf tube and placed on shaker at room temperature for 30 minutes. PBS to was  
25 added to 1 ml and the mixture was spun down at 13,000 rpm for 1 minute and then washed twice more with 1 ml of PBS.

- 30 The mixture was then spun down at 13,000 rpm for 1 minute and the beads were resuspended in a 50  $\mu$ l PBS per sample. 50  $\mu$ l of biotinylated  $\alpha$ -IgG-4 -coated beads were added to each sample and the mixture was incubated on a rotary shaker at 4 °C overnight. The tube was then spun at 1000 rpm for 5 minutes at room temperature.

The beads then were washed with 10 ml of PBS, spun down, resuspended in 1 ml of PBS, transferred to a sterile Eppendorf tube, washed with a further 2 x 1 ml of PBS, spun down and resuspended beads in a final volume of 250  $\mu$ l of DMEM plus 10%(HI)FCS plus glutamine plus P/S, i.e. at  $1.0 \times 10^5$  beads/ $\mu$ l.

5

CHO-N2 (clone N27) cells transfected with 10 x CBF1-Luc ( $T_{80}$  flask) were removed using 0.02% EDTA solution (Sigma), spun down and resuspended in 10 ml DMEM plus 10%(HI)FCS plus glutamine plus P/S. 10  $\mu$ l of cells were counted and the cell density was adjusted to  $1.0 \times 10^5$  cells/ml with fresh DMEM plus 10%(HI)FCS plus glutamine plus P/S.  $1.0 \times 10^5$  of the CHO-N2 cells transfected with 10xCBF1-Luc were plated out per well of a 24-well plate in a 1 ml volume of DMEM plus 10%(HI)FCS plus glutamine plus P/S and cells were placed in an incubator to settle down for at least 30 minutes.

15 100  $\mu$ l of beads were then added in duplicate to the first pair of wells to give  $1.0 \times 10^7$  beads / well (100 beads / cell); 20  $\mu$ l of beads added in duplicate to the second pair of wells to give  $2.0 \times 10^6$  beads / well (20 beads / cell); 4  $\mu$ l of beads added in duplicate to the third pair of wells to give  $4.0 \times 10^5$  beads / well (4 beads / cell) and 0  $\mu$ l of beads added to the fourth pair of wells. The plate was left in a CO<sub>2</sub> incubator overnight.

20

#### Luciferase assay

Supernatant was then removed from all the wells, 150  $\mu$ l of PBS and 150  $\mu$ l of SteadyGlo luciferase assay reagent (Promega) were added and the resulting mixture left at room temperature for 5 minutes.

25

The mixture was then pipetted up and down 2 times to ensure cell lysis and the contents from each well were transferred into an Eppendorf tube, spun at 13,000 rpm for 1 minute and the cleared supernatant was transferred to a white 96-well OptiPlate™ (Packard), leaving the bead pellet behind. Luminescence was then read in a TopCount™ (Packard) counter.

30

#### Example 10



### **Dynabeads ELISA Assay Method For Detecting Notch Ligand Activity**

5 M450 Streptavidin Dynabeads were coated with anti-hamster-IgG1 biotinylated monoclonal antibody, anti-human-IgG4 biotinylated monoclonal antibody or both antibodies and rotated for 2 hours at room temperature.

10 Beads were washed three times with PBS (1ml). The anti-hamster-IgG1 beads were then further incubated with anti-CD3 $\epsilon$  chain monoclonal antibody, the anti-human-IgG4 beads were further incubated with Fc-Delta, and the double coated beads incubated with both anti-CD3 $\epsilon$  chain monoclonal antibody and Fc-Delta. Beads were rotated overnight at 4°C, washed three times with PBS (1ml) and resuspended.

15 T-cell assays were carried out with CD4<sup>+</sup> T-cells and the beads. Supernatants were removed after 72 hours and cytokines measured by ELISA as described in Example 3. Results are shown in Figure 13.

### **Example 11**

20 **Modulation of cytokine production by human CD4<sup>+</sup> T cells in the presence of Delta1-hIgG4 immobilised on Dynal microbeads.**

25 Human peripheral blood mononuclear cells (PBMC) were purified from blood using Ficoll-Paque separation medium (Pharmacia). Briefly, 28 ml of blood were overlaid on 21 ml of Ficoll-Paque separation medium and centrifuged at 18-20°C for 40 minutes at 400g. PBMC were recovered from the interface and washed 3 times before use for CD4<sup>+</sup> T cell purification.

30 Human CD4<sup>+</sup> T cells were isolated by positive selection using anti-CD4 microbeads from Miltenyi Biotech according to the manufacturer's instructions.

The CD4<sup>+</sup> T cells were incubated in triplicates in a 96-well-plate (flat bottom) at 10<sup>5</sup> CD4/well/200μl in RPMI medium containing 10% FCS, glutamine, penicillin, streptomycin and β<sub>2</sub>-mercaptoethanol.

- 5 Cytokine production was induced by stimulating the cells with anti-CD3/CD28 T cell expander beads from Dynal at a 1:1 ratio (bead/cell) or plate bound anti-CD3 (clone UCHT1, BD Biosciences, 5μg/ml) and soluble anti-CD28 (clone CD28.2, BD Biosciences, 2μg/ml). Beads coated with mouse Delta1EC domain-hIgG4 fusion protein (prepared as described above with the modifications that incubation with
- 10 human IgG4 was for 30-40 minutes at room temperature and incubation with Delta-Fc was for two hours at room temperature) or control beads were added in some of the wells at a 10:1 ratio (beads/cell). The supernatants were removed after 3 or 4 days of incubation at 37°C/ 5%CO<sub>2</sub>/humidified atmosphere and cytokine production was evaluated by ELISA using Pharmingen kits OptEIA Set human IL10 (catalog No.
- 15 555157), OptEIA Set human IL-5 (catalog No. 555202) and OptEIA Set human IFNγ (catalog No 555142) for IL-10, IL-5 and IFNγ respectively and a human TNFα DuoSet from R&D Systems (catalog. No. DY210) for TNFα according to the manufacturer's instructions.
- 20 Results are shown in Figures 14 to 18.

### **Example 12**

#### **Variation of bead:cell ratios**

25

The procedure of Example 11 was repeated except that the ratio of control beads to cells and mouse Delta1-hIgG4 fusion protein coated beads to cells was varied between 16:1 and 0.25:1 (variously 16:1, 8:1, 4:1, 2:1, 1:1, 0.5:1, 0.25:1) and human Delta1-hIgG4 fusion protein coated beads were also used at the same ratios for comparison.

30

Results are shown in Figure 19.

**Example 13****Comparison of CD45RO+ (memory cells) and CD45RO- (naive cells)**

- 5 The procedure of Example 11 was repeated except that prior to the stimulation the human CD4+ were separated into CD45RO+ (memory cells) and CD45RO- (naive cells, data not shown on the slide). The magnetic separation was done using anti-CD4 Multisort microbeads (cat.No. 551-01) and then anti-CD45RO microbeads (cat.No.460-01) supplied by Miltenyi Biotec and following Miltenyi's protocol.

10

Results are shown in Figure 20.

**Example 14**

- 15 **Measurement of cytokine production in stimulated mouse CD4+ cells under polarising conditions**

**(i) CD4+ cell purification**

- 20 Spleens were removed from mice (variously Balb/c females, 8-10 weeks, C57B/6 females, 8-10 weeks, CARD1 females, 8-10 weeks (D011.10 transgenic, CAR transgenic)) and passed through a 0.2µm cell strainer into 20ml R10F medium (R10F-RPMI 1640 media (Gibco Cat No 22409) plus 2mM L-glutamine, 50µg/ml Penicillin, 50µg/ml Streptomycin,  $5 \times 10^{-5}$  M β-mercapto-ethanol in 10% fetal calf serum). The
- 25 cell suspension was spun (1150rpm 5min) and the media removed.

- The cells were incubated for 4 minutes with 5ml ACK lysis buffer (0.15M NH<sub>4</sub>Cl, 1.0M KHCO<sub>3</sub>, 0.1mM Na<sub>2</sub>EDTA in double distilled water) per spleen (to lyse red blood cells). The cells were then washed once with R10F medium and counted. CD4+
- 30 cells were purified from the suspensions by positive selection on a Magnetic Associated Cell Sorter (MACS) column (Miltenyi Biotec, Bisley, UK: Cat No 130-

042-401) using CD4 (L3T4) beads (Miltenyi Biotec Cat No 130-049-201), according to the manufacturer's directions.

(ii) Antibody Coating

5

96 well flat-bottomed plates were coated with Dulbecco's Phosphate Buffered Saline (DPBS) plus 1µg/ml anti-CD3 antibody (Pharmingen, San Diego, US: Cat No 553058, Clone No 145-2C11) plus 1µg/ml anti-IgG4 antibody (Pharmingen Cat No 555878). 100µl of coating mixture was used per well. Plates were incubated overnight at 4°C then washed with DPBS. Each well then received either 100µl DPBS or 100µl DPBS plus 10µg/ml Notch ligand (mouse Delta 1 extracellular domain/Ig4Fc fusion protein; Fc-delta). The plates were incubated for 2-3 hours at 37°C then washed again with DPBS before cells (prepared as in (i)) were added.

15 (iii) Primary Polyclonal Stimulation

CD4+ cells were cultured in 96 well, flat-bottomed plates pre-coated as in (ii) above. Cells were re-suspended, following counting, at  $2 \times 10^6$  /ml in R10F medium plus 4µg/ml anti-CD28 antibody (Pharmingen, Cat No 553294, Clone No 37.51). 100µl cell suspension was added per well. 100µl of polarising or control medium was then added to each well to give a final volume of 200µl ( $2 \times 10^5$  cells/well, anti-CD28 final concentration 2µg/ml) as follows:

Un-polarised cells: R10F medium.

25 Th1 polarised cells: R10F medium plus anti-IL-4 antibody (10µg/ml, Pharmingen Cat No 554432) plus IL-12 (10ng/ml, Peprotech 210-12).

Th2 polarised cells: R10Fmedium plus anti-IL-12 antibody (10µg/ml, Pharmingen Cat No 554475) plus anti-IFNγ antibody (1µg/ml, Pharmingen Cat No 554408) plus IL-4 (10ng/ml, Peprotech Cat No 214-14).

30

The plates were then incubated at 37°C for 72 hours.

125µl supernatant was then removed from each well and stored at -20°C until tested by ELISA for IL-10 and TNFα using antibody pairs from R & D Systems (Abingdon, UK). The cells were then split 1 in 3 into new wells (not coated) and fed with R10F medium plus recombinant human IL-2 (2.5ng/ml, PeproTech Inc, London, UK: Cat No 200-02).

Results are shown in Figure 21.

10

### **Example 15**

#### **Gene expression profiling**

##### **(i) Cell culture, treatments and RNA extraction**

Jurkat cells were cultured in RPMI 1640 (GibcoBRL) supplemented with 2mM Glutamine (GibcoBRL), Penicillin-Streptomycin 50 units/ml (GibcoBRL) and with 10% Fetal Bovine Serum (FBS) (Biochrom KG).

20

Anti-V5 (Invitrogen) and anti-CD3 (human), anti-CD28 (human) antibodies (PharMingen) were plated at 5µg/ml in phosphate buffer saline (Gibco BRL) in 6 well tissue culture dishes (1ml PBS/well) overnight. Anti-V5 antibody was applied to every well, while mouse IgG<sub>1</sub> κ isotype control at 10 µg/ml was applied in wells that no anti-CD3 or anti-CD28 was used. The next day the wells were washed 3 times with PBS, and Delta-V5-His protein was plated at 5µg/ml PBS (1ml/well). The plates were then incubated at 37°C for 2 hours and then washed with PBS three times. Jurkat cells were then plated out at a concentration of  $2 \times 10^6$  cells /ml and incubated at 37°C. Ionomycin was added to the appropriate wells at a concentration of 1µg/ml (Sigma). Cells were taken out at 2, 4, 8, 18, 24, 36, 48 hrs, washed once with PBS at 4°C and collected at 300-600 µl RLT lysis solution (Qiagen). In order to ensure the efficacy of the stimulation, cells were tested for the correct expression of T cell activation markers

30

using FACs analysis. The cells used in this experiment were all expressing CD69 (early activation marker) after 48h of anti-CD3, anti-CD28 activation.

RNA was extracted using an RNA Easy miniprep kit (Qiagen) according to the manufacturer's instructions. The optional DNase step recommended was also performed. A phenol extraction step was performed to ensure the complete lack of proteins in the RNA. RNA was then amplified using the MessageAmp aRNA Kit (Ambion) following the manufacturer's recommendations. Briefly, the procedure consists of reverse transcription with an oligo(dT) primer bearing a T7 promoter and in vitro transcription of the resulting DNA with T7 RNA polymerase to generate hundreds of thousands of antisense RNA (αRNA) copies of each mRNA in the sample.

The nomenclature used was as follows: RNA from cells that were plated on wells treated only with V5 was labelled 'V5', while RNA from cells plated on wells treated with anti-V5 and Delta-V5-His was labelled 'Delta'. RNA from cells plated on wells treated with anti-V5, anti-CD3, anti-CD28 were labelled 'CD3CD28' while RNA from cells plated on wells treated with anti-V5, anti-CD3, anti-CD28, Delta-V5-His was labelled 'CD3CD28Delta'. Similarly RNA from cells plated on anti-V5 and further treated with ionomycin was labelled 'ionomycin' while RNA from cells plated on anti-V5, Delta-V5-His and further treated with ionomycin were labelled 'ionomycin-Delta'.

### **(ii) Gene Expression Profiling**

Microarrays were manufactured by spotting purified PCR products onto glass slides. Microarray probes were prepared by labelling 2μg of αRNA by a reverse transcriptase reaction incorporating dCTP-Cy3 or dCTP-Cy5 labelled nucleotide. Probe labelling and purification were then performed generally as described in Hegde P, Qi R, Abernathy K, Gay C, Dharap S, Gaspard R, Hughes JE, Snesrud E, Lee N, Quackenbush J: A concise guide to cDNA microarray analysis (2000). *Biotechniques* 29:548-50, 552-4, 556 passim.

Purified probes were then hybridized on the arrays overnight at 42°C in 10 x SSC, 50% formamide, 0.2% SDS solution. Slides were then washed twice in 2 x SSC, 0.2% SDS for 7 min at 42°C, twice in 0.1 SSC/ 0.2% SDS for 5 minutes at room temperature, and finally once in 0.2%SSC at room temperature. For each time point the sample named 'V5' was labelled with dCTP-Cy3 and hybridized on the same slide as the sample named 'Delta' that was labelled with dCTP-Cy5. Similarly the sample named CD3CD28 was labelled with dCTP-Cy3 and hybridized on the same slide as the sample named 'CD3CD28Delta' that was labelled with dCTP-Cy5. Finally the sample named 'ionomycin' was labelled with dCTP-Cy3 and hybridized on the same slide as the sample labelled 'ionomycinDelta' that was labelled with dCTP-Cy5 (see Table-1).

**Table-1**

	<b>Label 1 (Cy3-dCTP)</b>	<b>Label 2 (Cy5-dCTP)</b>
Slide	V5	Delta
Slide	CD3CD28	CD3CD28Delta
Slide	Ionomycin	IonomycinDelta

Once dried the slides were scanned on a GSI Lumonics confocal scanner at 100% laser power and 65-75% photo-multiplier tube efficiency (depending on background). Slide images were processed as follows: Array spots representing the signal associated with individual spotted clones were identified and quantified using the Quantarray application (GSI Lumonics). Numeric values for the gene expression intensities were calculated using the histogram method implemented in the same application. Values were calculated as integrals of the pixel signal distribution associated to each spot and local background values subtracted (raw data).

### **(iii) Data Processing**

For all data analyses the GeneSpring package (Silicon Genetics) was used. Raw data from Quantarray was introduced in GeneSpring, and the ratio between the signal and control intensities was calculated for each gene at each time point. Intensities for genes

from samples labelled 'Delta' or 'CD3CD28Delta', or 'ionomycinDelta' were regarded as 'signals' while the intensities from genes from samples labelled either 'V5' or 'CD3CD28' or 'ionomycin' were regarded as 'controls'.

- 5     Ratio= signal strength of gene in 'Delta'/ control strength of gene in 'V5'  
Ratio= signal strength of gene in 'CD3CD28Delta'/ control strength of gene in  
      'CD3CD28'  
Ratio= signal strength of gene in 'ionomycinDelta'/ control strength of gene in  
      'ionomycin'

10

When this ratio was  $>2$  the gene was considered to be upregulated, while when the ratio was  $<0.5$  the ratio the gene was considered to be downregulated.

- 15     A schematic representation of the protocol for activating with Delta alone and a Venn diagram showing numbers of genes showing increased expression in response to Delta activation alone are shown in Figures 22A and 22B respectively, and a corresponding time-course expression profile is shown in Figure 23.

- 20     A schematic representation of the protocol for activating with both Delta and anti-CD3/CD28 activation and a Venn diagram showing numbers of genes showing increased expression in response to Delta activation in combination with anti-CD3/CD28 activation but not Delta activation alone are shown in Figures 24A and 24B respectively, and a corresponding time-course expression profile is shown in Figure 25.

25

- 30     All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out



the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

References

- Tamura et al. (1995) *Curr. Biol.* **5**:1416-1423.
- 5 Artavanis-Tsakomas et al. (1995) *Science* **268**:225-232.  
Artavanis-Tsakomas et al. (1999) *Science* **284**:770-776.  
Lieber et al. (1993) *Genes Dev* **7**(10):1949-65.  
Schroeter et al. (1998) *Nature* **393**(6683):382-6.  
Struhl et al. (1998) *Cell* **93**(4):649-60.
- 10 Weinmaster(2000) *Curr. Opin. Genet. Dev.* **10**:363-369.  
Lu et al. (1996) *Proc Natl Acad Sci* **93**(11):5663-7.  
Munro and Freeman (2000) *Curr. Biol.* **10**:813-820.  
Ju et al. (2000) *Nature* **405**:191-195.  
Moloney et al. (2000) *Nature* **406**:369-375.
- 15 Brucker et al. (2000) *Nature* **406**:411-415.  
Panin et al. (1997) *Nature* **387**:908-912.  
Hicks et al. (2000) *Nat. Cell. Biol.* **2**:515-520.  
Irvine (1999) *Curr. Opin. Genet. Devel.* **9**:434-441.  
Devereux et al. (1984) *Nucleic Acid Research* **12**:87.
- 20 Atschul et al. (1990) *J. Mol. Biol.* **403**:410.  
Inaba et al. (1992) *J. Exp. Med.* **175**:1157-1167.  
Caux et al. (1992) *Nature* **360**:258-261.  
Coffin et al. (1998) *Gene Therapy* **5**:718-722.  
Kroll et al. (1993) *DNA Cell Biol.* **12**:441-453.
- 25 Osborne and Miele (1999) *Immunity* **11**:653-663.  
Matsuno et al. (1995) *Development* **121**(8):2633-44.  
Matsuno et al. (1998) *Nat. Genet.* **19**:74-78.
- Ordentlich et al. (1998) *Mol. Cell. Biol.* **18**:2230-2239.  
Takebayashi et al. (1994) *J Biol Chem* **269**(7):150-6.
- 30 Leimeister et al. (1999) *Mech Dev* **85**(1-2):173-7.  
Maddox (1983) *J. Exp. Med.* **15**(8):121 1

Sauber et al (1995) *Virology* **213**:439-449

Chee et al. (1996) *Science* **274**:601-614.

Camilli et al. (1994) *Proc Natl Acad Sci USA* **91**:2634-2638.

Hoyne et al. (2000) *Immunology* **100**:281-288.

5 Hoyne et al. (2001) – reference in press.

CLAIMS

1. A method for detecting modulators of Notch signalling comprising the steps of monitoring Notch signalling in a cell of the immune system in the presence and  
5 absence of a candidate modulator, and determining whether the candidate modulator modulates Notch signalling.
2. A method for detecting modulators of Notch signalling comprising the steps of:
  - (a) contacting a cell of the immune system with a candidate modulator;
  - 10 (b) monitoring Notch signalling; and
  - (c) determining whether the candidate modulator modulates Notch signalling.
3. A method according to claim 1 or claim 2 wherein the candidate modulator is selected from the group consisting of: an organic compound, a inorganic compound, a  
15 peptide or polypeptide, a polynucleotide, an antibody, a fragment of an antibody, a cytokine and a fragment of a cytokine.
4. A method according to any preceding claim wherein the step of monitoring Notch signalling comprises the step of monitoring levels of expression of at least one  
20 target gene.
5. A method according to claim 4 wherein the at least one target gene is an endogenous target gene of Notch signalling.
- 25 6. A method according to claim 5 wherein the at least one target gene is selected from the group consisting of: Deltex, Hes-1, E(spl), Il-10, CD-23, Dlx-1, CTLA4, CD-4, Numb, Mastermind and Dsh.
7. A method according to claim 4 wherein the at least one target gene is a reporter  
30 gene.

8. A method according to claim 7 wherein the at least one target gene is selected from the group consisting of: a gene encoding a polypeptide having an enzymatic activity, a gene comprising a radiolabel or a fluorescent label and a gene encoding a predetermined polypeptide epitope.

5

9. A method according to any one of claims 4 to 8 wherein the at least one target gene is under the transcriptional control of a promoter region sensitive to Notch signalling.

10 10. A method according to any of claims 1 to 8 wherein the at least one target gene is under the transcriptional control of a promoter region sensitive to:

- i) Notch signalling; and
- ii) a second signal; and/or
- iii) a third signal

15 wherein the second and third signals are different.

11. A method according to claim 10 wherein the second signal results from activation of a signalling pathway specific to cells of the immune system.

20 12. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a T cell receptor (TCR) signalling pathway.

13. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a B cell receptor (BCR) signalling pathway.

25

14. A method according to claim 11 wherein the signalling pathway specific to cells of the immune system is a Toll-like receptor (TLR) signalling pathway.

15. A method according to any one of claims 10 to 14 wherein the third signal is a  
30 costimulus specific to cells of the immune system.

16. A method according to claim 15 wherein the costimulus is selected from the group consisting of: B7 proteins B7.1-CD80, B7.2-CD86, B7H1, B7H2, B7H3, B7RP1, B7RP2, CTLA4, ICOS, CD2, CD24, CD27, CD30, CD34, CD38, CD40, CD44, CD45, CD49, CD69, CD70, CD95 (Fas), CD134, CD134L, CD153, CD154, 4-1BB, 4-1BB-L, LFA-1, ICAM-1, ICAM-2, ICAM-3, OX40, OX40L, TRANCE/RANK ligands, Fas ligand, MHC class II, DEC205-CD205, CD204-Scavenger receptor, CD14, CD206 (mannose receptor), Toll-like receptors (TLRs), such as TLR 1-9, CD207 (Langerin), CD209 (DC-SIGN), FC $\gamma$  receptor 2 (CD32), CD64 (FC $\gamma$  receptor 1), CD68, CD83, CD33, CD54, BDCA-2, BDCA-3, BDCA-4, chemokine receptors, cytokines, growth factors and growth factor receptor agonists, and variants, derivatives, analogues and fragments thereof.
17. A method according to any preceding claim wherein the cell of the immune system is a T cell or T cell progenitor.
18. A method according to any one of claims 1 to 16 wherein the cell of the immune system is an antigen presenting cell (APC).
19. A method according to any one of claims 4 to 18 wherein expression of the at least one target gene is monitored with a protein assay.
20. A method according to any of claims 4 to 18 wherein expression of the at least one target gene is monitored with a nucleic acid assay.
21. A modulator identifiable by a method according to any one of the preceding claims.
22. Use of a modulator according to claim 21 for the preparation of a medicament for the treatment of a disease or condition of, or related to, the immune system.

23. Use of a modulator according to claim 22 wherein the disease is a T-cell mediated disease.
24. Use of a modulator according to claim 22 wherein the disease is a B-cell mediated disease.
25. Use of a modulator according to claim 22 wherein the disease is an APC mediated disease.
26. A pharmaceutical composition comprising a therapeutically effective amount of at least one modulator according to claim 22 and a pharmaceutically acceptable carrier, diluent and/or excipient.
27. A method for detecting modulators of Notch signalling comprising the steps of:
- (a) activating a cell of the immune system;
  - (b) contacting the cell with a candidate modulator;
  - (c) monitoring Notch signalling;
- (wherein steps (a), (b) and (c) can be carried out in any order); and
- (d) determining whether the candidate modulator modulates Notch signalling.
28. A method as claimed in claim 27 wherein the cell of the immune system is a T-cell.
29. A method as claimed in claim 28 wherein the T-cell is activated by activation of the T-cell receptor.
30. A method as claimed in claim 29 wherein the T-cell receptor is activated with an antigen or antigenic determinant.
31. A method as claimed in claim 29 wherein the T-cell receptor is activated by an anti-CD3 antibody

32. A method as claimed in any one of claims 28 to 31 wherein the T-cell is co-activated
- 5 33. A method as claimed in claim 32 wherein the T-cell is co-activated by activation of CD28.
34. A method as claimed in claim 33 wherein the T-cell receptor is co-activated by an anti-CD28 antibody.
- 10 35. A method as claimed in claim 34 wherein the anti-CD28 antibody is bound to a support.
36. A method as claimed in claim 35 wherein the anti-CD28 antibody is bound to a
- 15 particulate support.
37. A method as claimed in any one of claims 32 to 36 wherein the T-cell is activated by an anti-CD3 antibody and co-activated by anti-CD28 antibody.
- 20 38. A method as claimed in any one of claims 31 to 39 comprising the steps of:  
i) activating Notch signalling in the immune cell with a further agent; and  
ii) determining whether the candidate modulator modulates such Notch signalling activation and/or immune cell activation.
- 25 39. A method as claimed in claim 38 wherein Notch signalling is activated with a Notch ligand.
40. A method as claimed in claim 39 wherein the Notch ligand is bound to a support.
- 30 41. A particle comprising a modulator of Notch signalling bound to a particulate support matrix.



42. A particle as claimed in claim 41 wherein the particulate support matrix is a bead.
43. A particle as claimed in claim 41 or claim 42 wherein the modulator of Notch signalling is a Notch ligand.
44. A particle as claimed in claim 43 wherein a plurality of Notch ligands are bound to the particulate support matrix.
45. A method for detecting modulators of Notch signalling substantially as hereinbefore described with reference to the accompanying figures.
46. A modulator substantially as hereinbefore described with reference to the accompanying figures.
47. A particle substantially as hereinbefore described with reference to the accompanying figures.
48. Use of a modulator substantially as hereinbefore described with reference to the accompanying figures.
49. A pharmaceutical composition substantially as hereinbefore described with reference to the accompanying figures.
50. A method for detecting modulators of Notch signalling comprising the steps of:
- (a) contacting an immune cell with a candidate modulator;
  - (b) monitoring gene expression; and
  - (c) determining whether the candidate modulator modulates gene expression.
51. A method for detecting modulators of Notch signalling comprising the steps of:
- (a) activating a cell of the immune system;

- (b) contacting the cell with a candidate modulator;
- (c) monitoring gene expression;
- (wherein steps (a), (b) and (c) can be carried out in any order); and
- (d) determining whether the candidate modulator modulates gene expression.

5

52. A method for detecting genes which are upregulated or downregulated in an immune cell in response to a Notch signalling comprising the steps of:

- (a) contacting the cell with a known Notch signalling modulator;
- (b) monitoring gene expression;
- 10 (wherein steps (a) and (b) can be carried out in any order); and
- (c) determining which genes are upregulated or downregulated.

53. A method for detecting genes which are upregulated in an immune cell in response to a combination of Notch signalling and immune cell activation comprising  
15 the steps of:

- (a) activating an immune cell;
- (b) contacting the cell with a known Notch signalling modulator;
- (c) monitoring gene expression;
- (wherein steps (a), (b) and (c) can be carried out in any order); and
- 20 (d) determining which genes are upregulated or downregulated.

54. A method for detecting genes which are upregulated or downregulated in an immune cell in response to a combination of Notch signalling and immune cell activation but not in response to Notch signalling or immune cell activation alone  
25 comprising the steps of:

- (a) activating an immune cell;
- (b) contacting the cell with a Notch signalling modulator;
- (c) monitoring gene expression;
- (wherein steps (a), (b) and (c) can be carried out in any order);

- (d) determining whether gene expression is upregulated or downregulated in the cell;
- (e) comparing gene expression from step (d) with controls in which the cell is not activated or the cell is not contacted with the Notch signalling modulator.

5

55. A method as claimed in any one of claims 50 to 54 wherein gene expression is monitored using a microarray.

10

56. A method as claimed in any one of claims 50 to 55 wherein the immune cell is a T-cell.

57. A gene detected by a method as claimed in any one of claims 50 to 56.

15

58. A method for modifying IL-5 expression in a cell by administering a modulator of Notch signalling.

59. A method for modifying IL-13 expression in a cell by administering a modulator of Notch signalling.

20

60. A method for modifying TNF expression in a cell by administering a modulator of Notch signalling.

61. A method for modifying IL-10 expression in a cell by administering a modulator of Notch signalling.

25

62. A method for reducing TNF expression in a cell by administering a modulator of Notch signalling.

63. A method for increasing IL-10 expression in a cell by administering a modulator of Notch signalling.
64. A method for reducing IL-5 expression in a cell by administering a modulator of Notch signalling.
65. A method for reducing IL-13 expression in a cell by administering a modulator of Notch signalling.
66. A method as claimed in any one of claims 58 to 65 wherein the modulator of Notch signalling modifies cytokine expression in leukocytes, fibroblasts or epithelial cells.
67. A method as claimed in claim 66 wherein the modulator of Notch signalling modifies cytokine expression in lymphocytes or macrophages.
68. A method for generating an immune modulatory cytokine profile with increased IL-10 expression and reduced TNF $\alpha$  expression by administering a modulator of Notch signalling.
69. A method for generating an immune modulatory cytokine profile with increased IL-10 expression and reduced IL-5 expression by administering a modulator of Notch signalling.
70. A method for generating an immune modulatory cytokine profile with increased IL-10 expression and reduced IL-13 expression by administering a modulator of Notch signalling.
71. A method for generating an immune modulatory cytokine profile with reduced IL-5, IL-13 and TNF $\alpha$  expression by administering a modulator of Notch signalling.

72. A method for generating an immune modulatory cytokine profile with reduced IL-2, IFN $\gamma$ , IL-5, IL-13 and TNF $\alpha$  expression by administering a modulator of Notch signalling.

5 73. A method as claimed in claim 71 or claim 72 wherein the cytokine profile exhibits increased IL-10 expression.

74. A method for reducing a TH2 immune response by administering a modulator of Notch signalling.

10

75. A method for reducing a TH1 immune response by administering a modulator of Notch signalling.

15 76. A method for treating inflammation or an inflammatory condition by administering a modulator of Notch signalling.

77. A method for treating inflammation or an inflammatory or autoimmune condition by administering a modulator of Notch signalling to reduce TNF $\alpha$  expression.

20 78. The use of a modulator of Notch signalling to modify TNF expression in a cell.

79. The use of a modulator of Notch signalling to modify IL-10 expression in a cell.

25 80. The use of a modulator of Notch signalling to modify IL-5 expression in a cell.

81. The use of a modulator of Notch signalling to reduce TNF expression in a cell.

82. The use of a modulator of Notch signalling to increase IL-10 expression in a cell.

30 83. The use of a modulator of Notch signalling to reduce IL-5 expression in a cell.

84. A use as claimed in any one of claims 78 to 83 wherein the modulator of Notch signalling modifies cytokine expression in leukocytes, fibroblasts or epithelial cells.

5 85. A use as claimed in claim 84 wherein the modulator of Notch signalling modifies cytokine expression in lymphocytes or macrophages.

86. The use of a modulator of Notch signalling to generate an immune modulatory cytokine profile by increasing IL-10 expression and reducing TNF $\alpha$  expression.

10 87. The use of a modulator of Notch signalling to generate an immune modulatory cytokine profile by increasing IL-10 expression and reducing IL-5 expression.

15 88. The use of a modulator of Notch signalling to generate an immune modulatory cytokine profile by increasing IL-10 expression and reducing IL-13 expression.

89. The use of a modulator of Notch signalling to generate an immune modulatory cytokine profile by reducing IL-5, IL-13 and TNF $\alpha$  expression.

20 90. The use of a modulator of Notch signalling to generate an immune modulatory cytokine profile by reducing IL-2, IFN $\gamma$ , IL-5, IL-13 and TNF $\alpha$  expression.

91. A use as claimed in claim 89 or claim 90 wherein IL-10 expression is increased.

25 92. The use of a modulator of Notch signalling to reduce a TH2 immune response.

93. The use of a modulator of Notch signalling to reduce a TH1 immune response.

30 94. The use of a modulator of Notch signalling in the manufacture of a medicament for the treatment of inflammation or an inflammatory condition.

95. The use of a modulator of Notch signalling in the manufacture of a medicament for the treatment of inflammation or an inflammatory or autoimmune condition by reduction of TNF $\alpha$  expression.

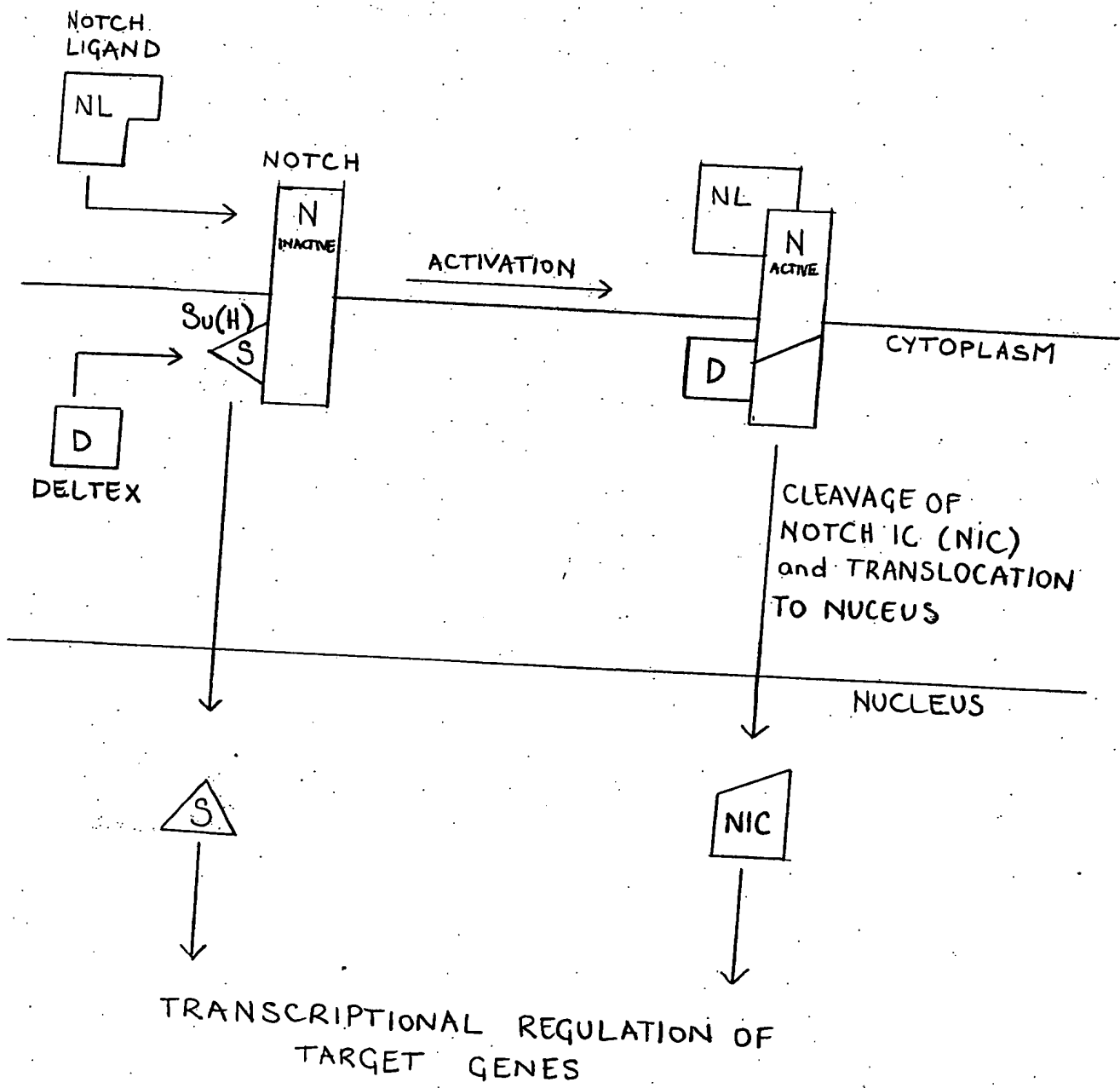
**ABSTRACT**

**A METHOD**

A method for detecting modulators of Notch signalling comprising the step of  
monitoring Notch signalling in a cell of the immune system in the presence of a  
5 candidate modulator.



FIGURE 1



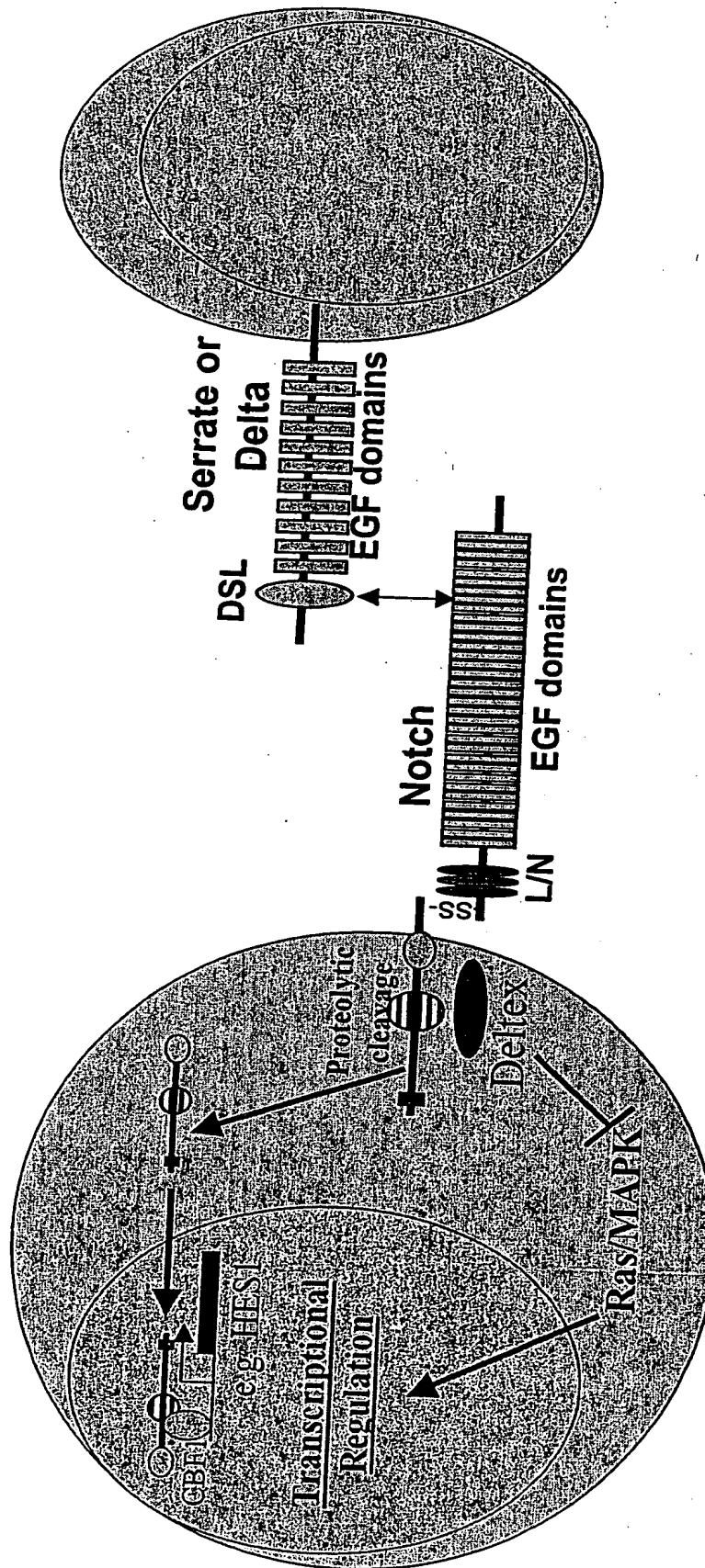
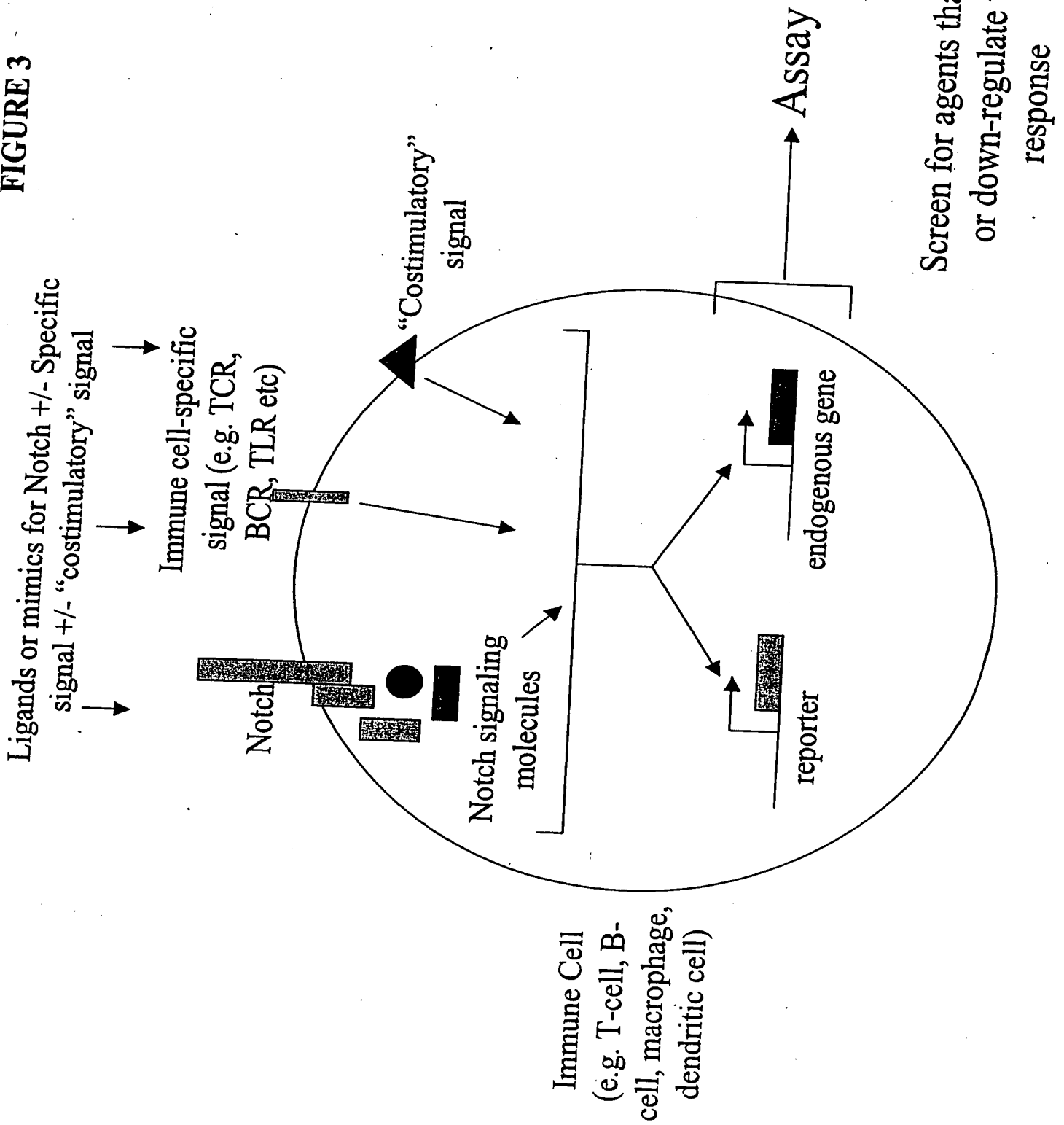
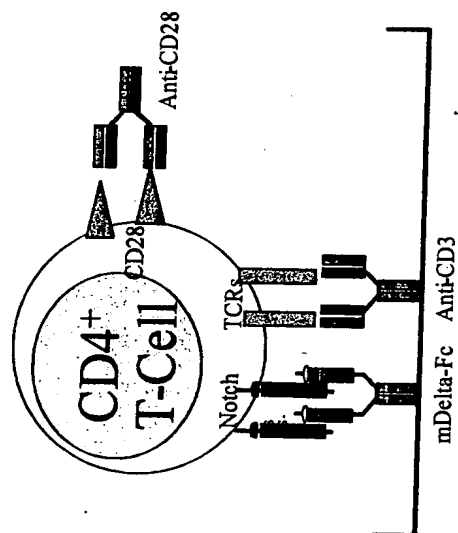
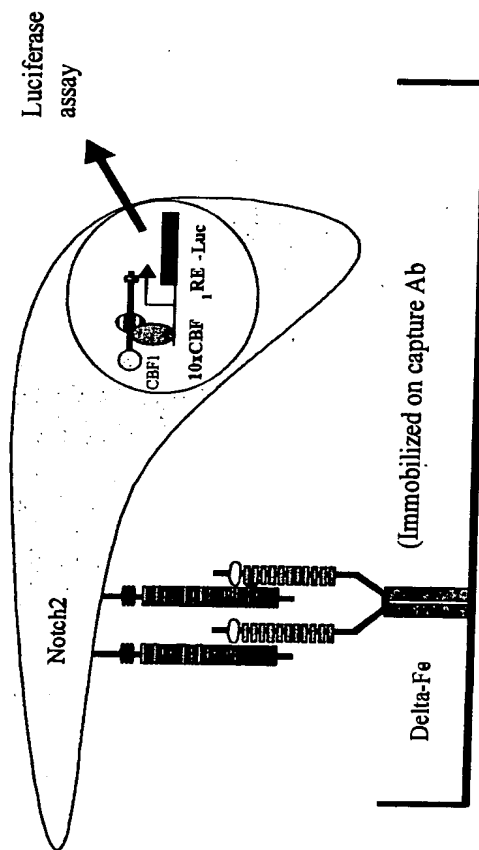


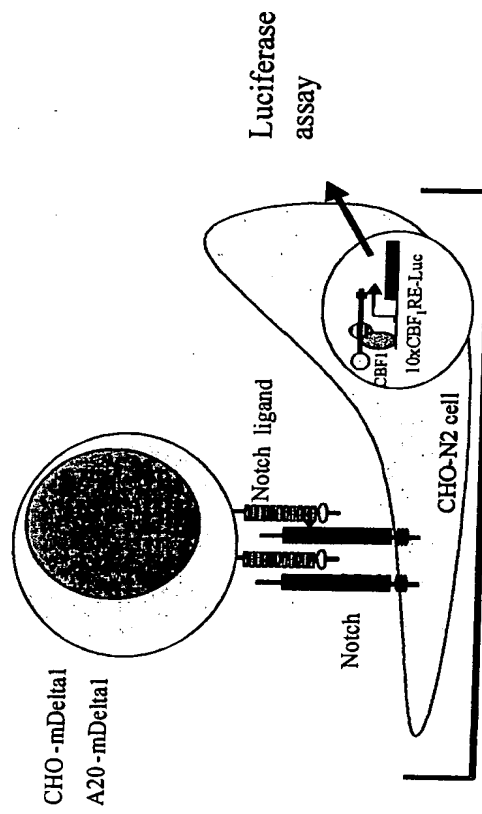
Figure 2

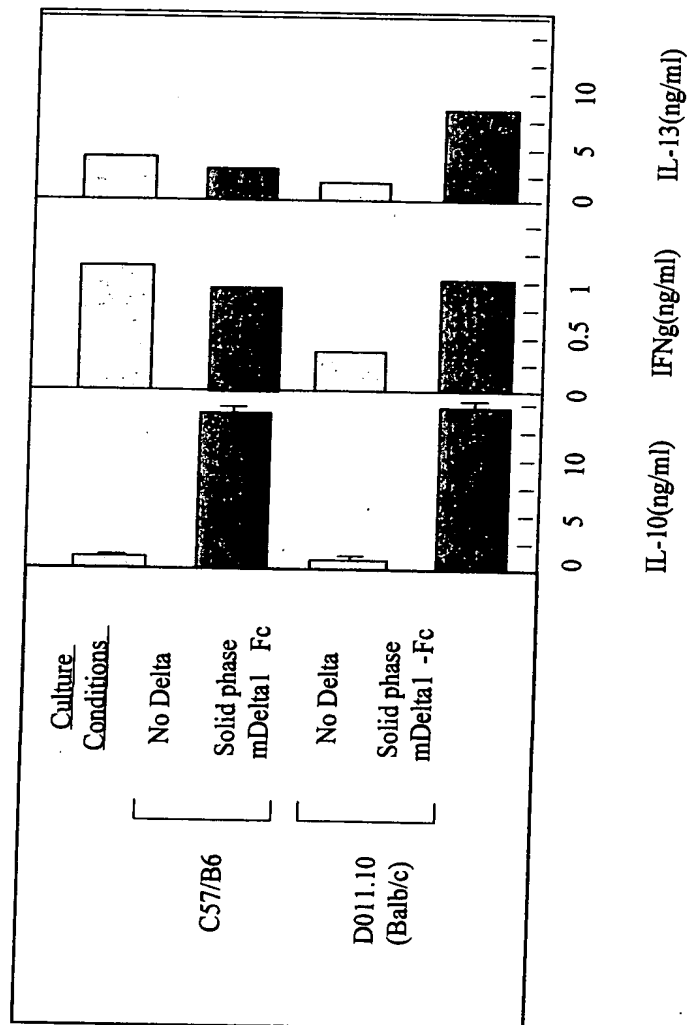
**FIGURE 3**

**Figure 4**

**Figure 5**

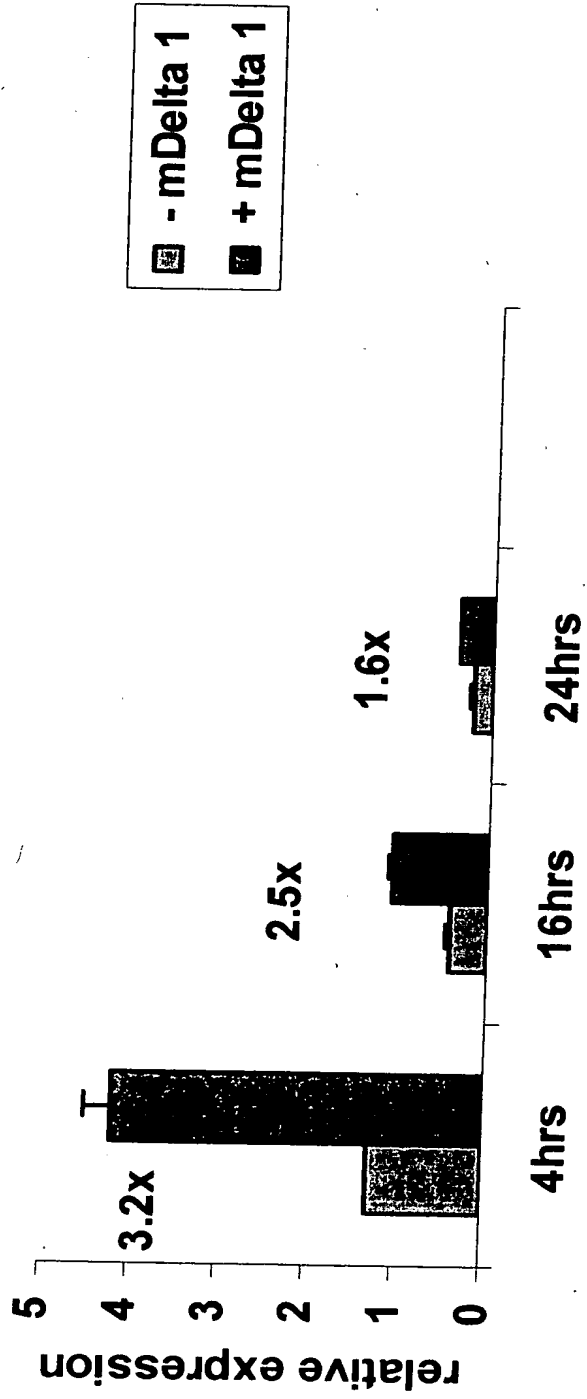


**Figure 6**

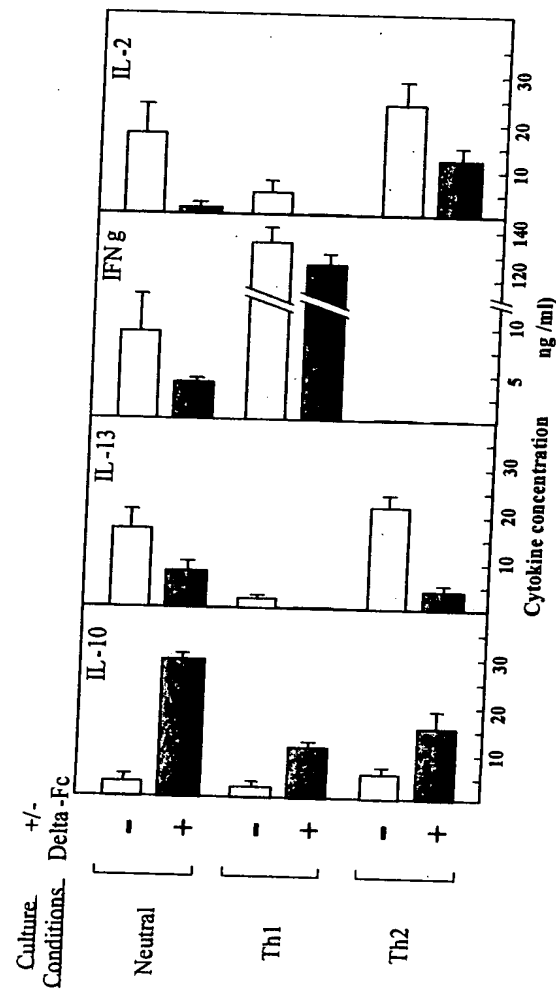
**Figure 7**

**Figure 8**

Relative expression of mHes1 in Cd4+ T cells





**Figure 9****Cytokine production under polarising conditions**

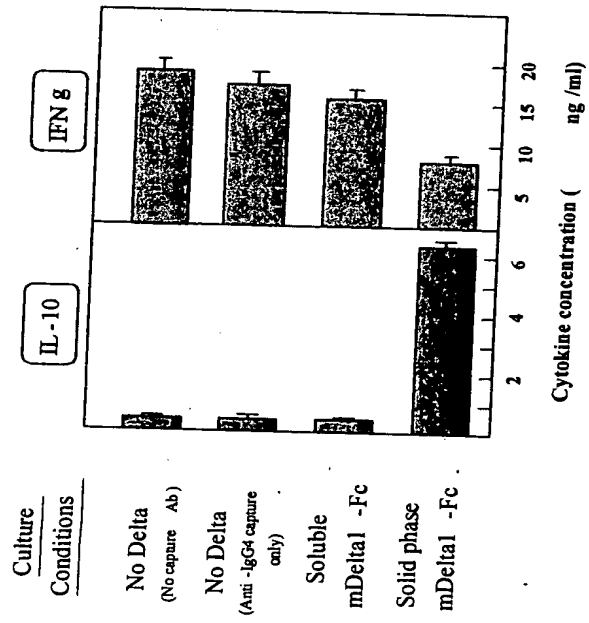
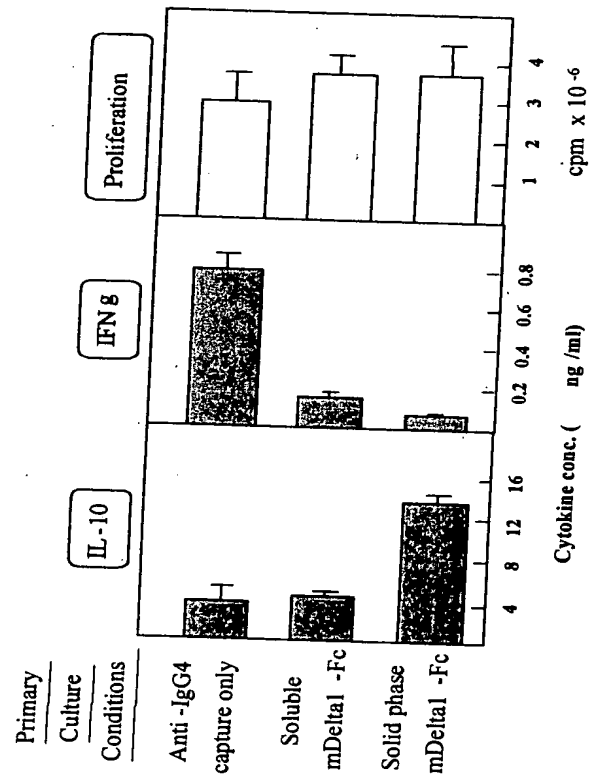
**Figure 10**

Figure 11



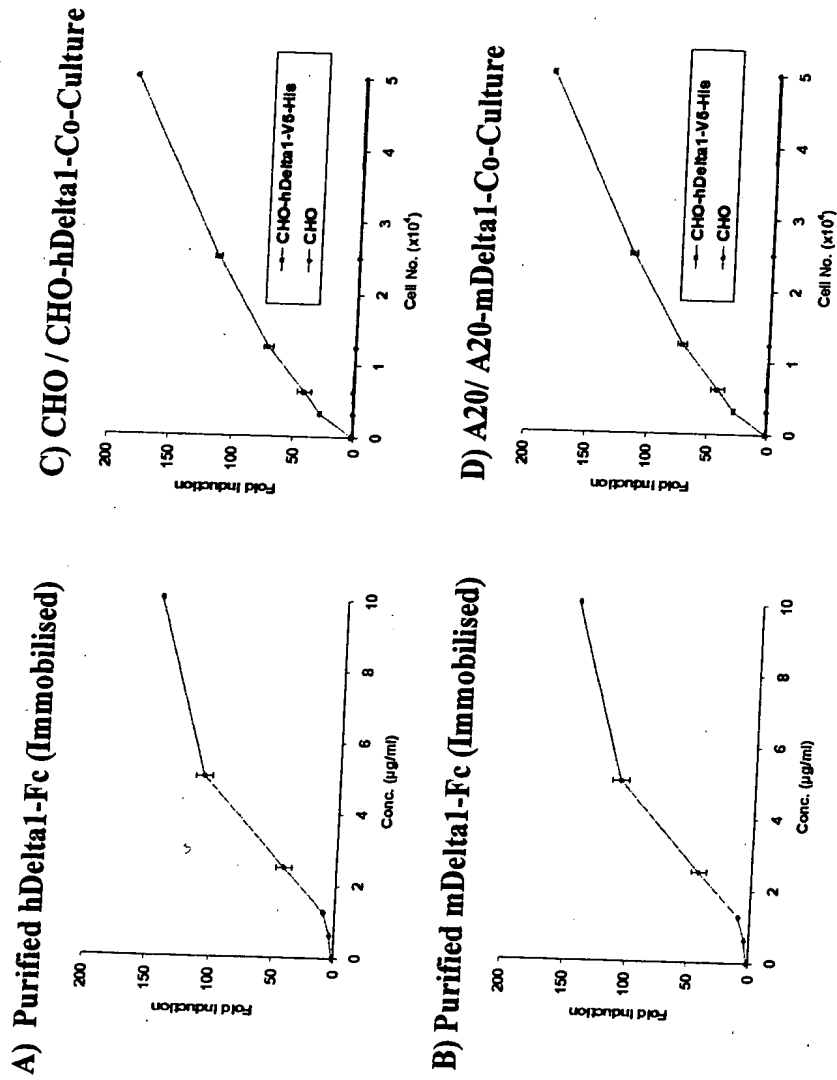
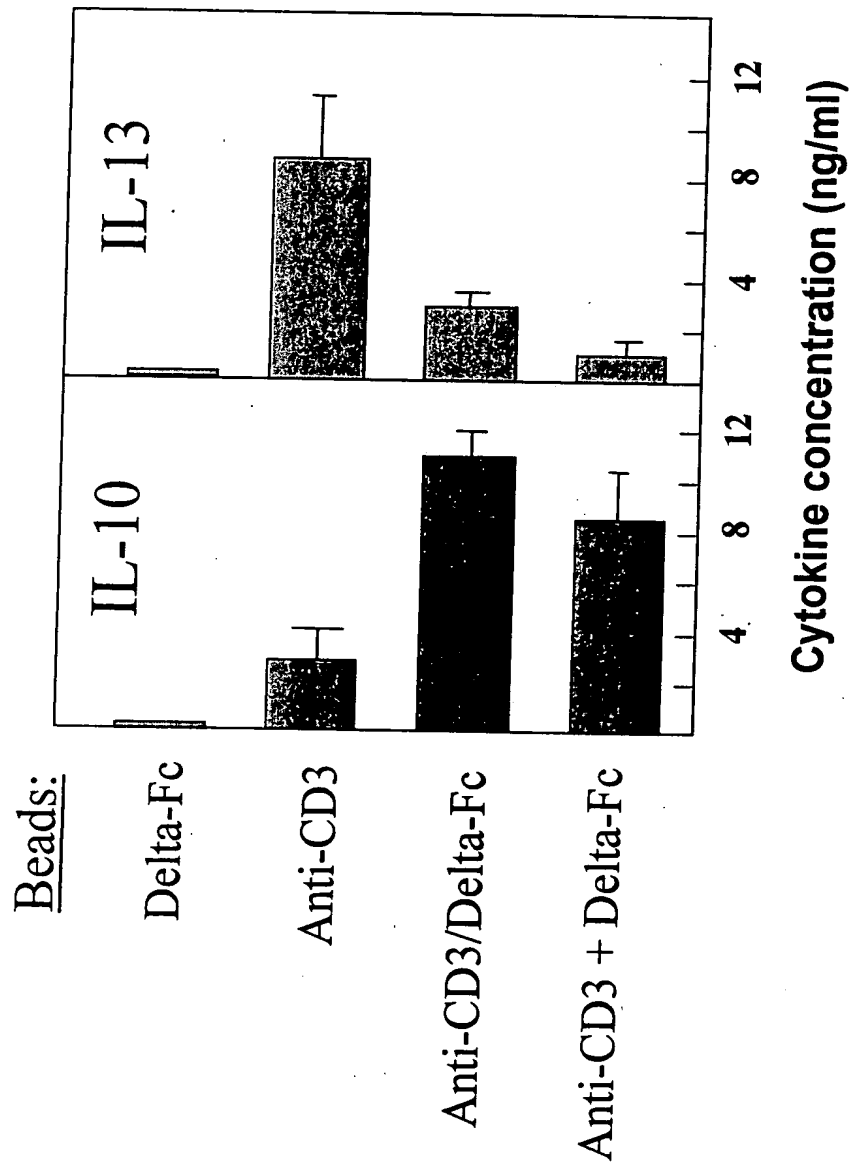
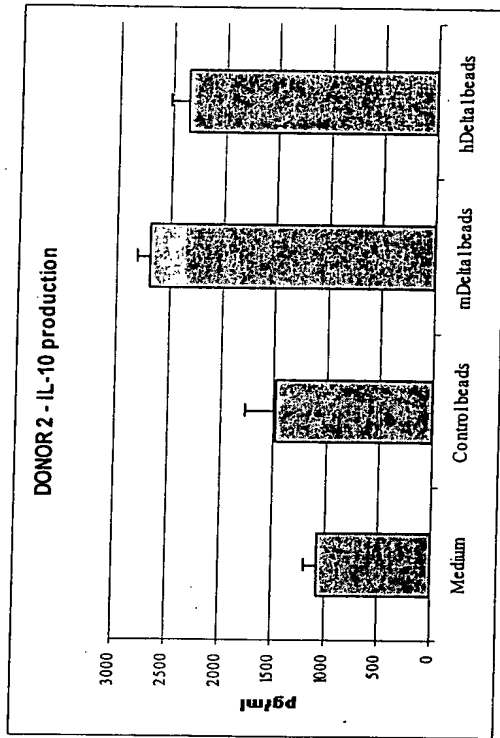
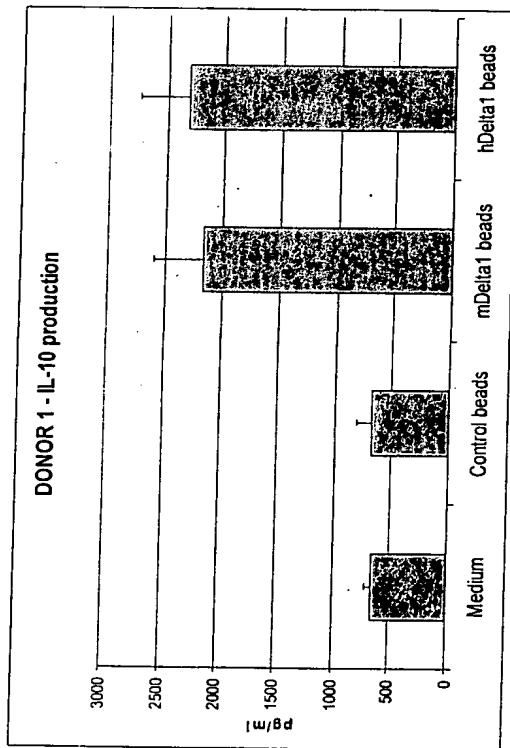
**Figure 12**

Figure 13: Delta-Fc coated beads modulate *in vitro* T-cell responses

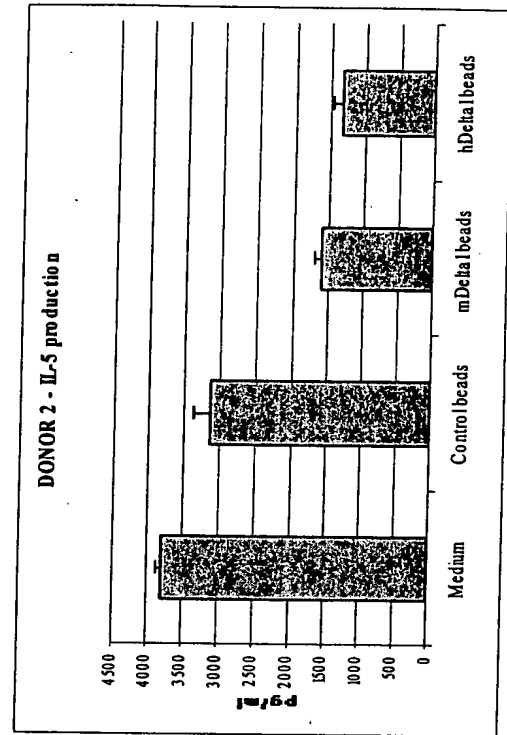
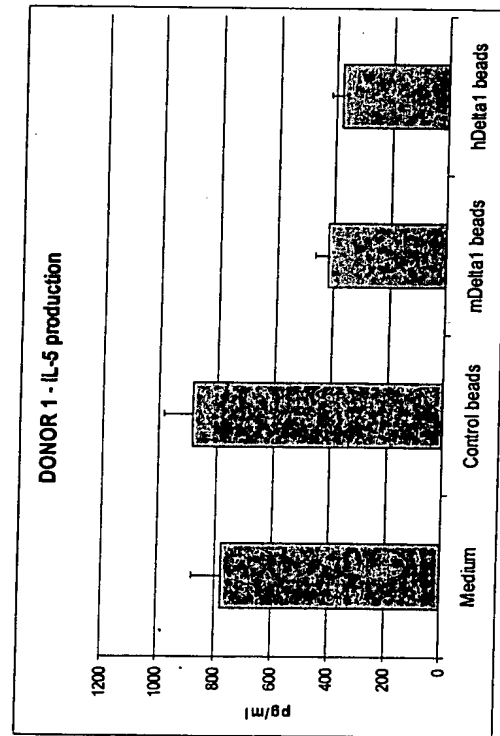


CD4+ T-cells activated with beads coated as described plus soluble anti-CD28, 3d

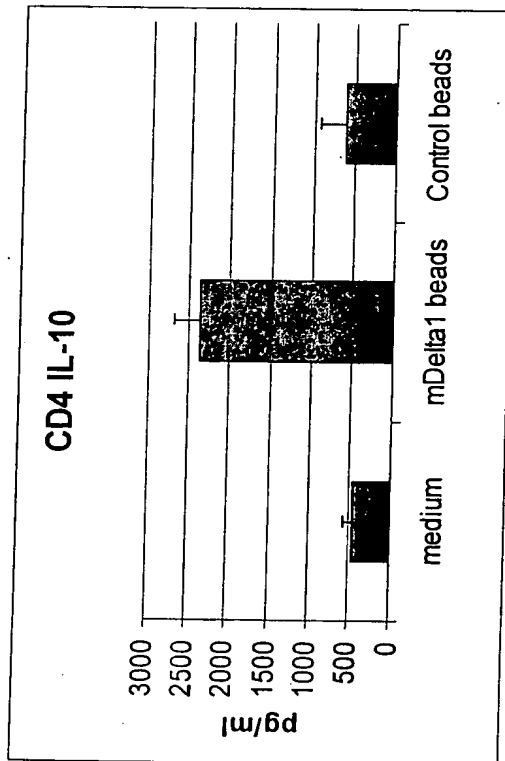
**Figure 14: Increase in IL-10 production in the presence of mouse or human Delta1 beads**



**Figure 15: Decrease in IL-5 production in the presence of mouse or human Delta1 beads**



**Figure 16:** Increase in IL-10 production in the presence of mouse Delta1 beads



**Figure 17:** Decrease in IL-5 production in the presence of mouse Delta1 beads

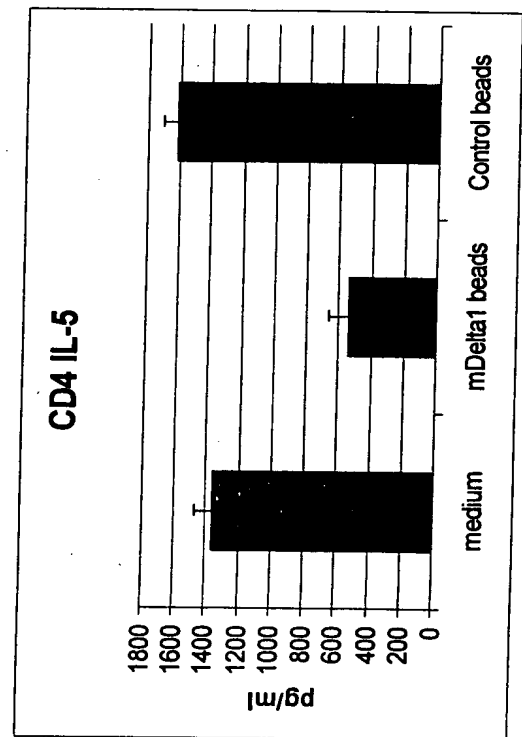
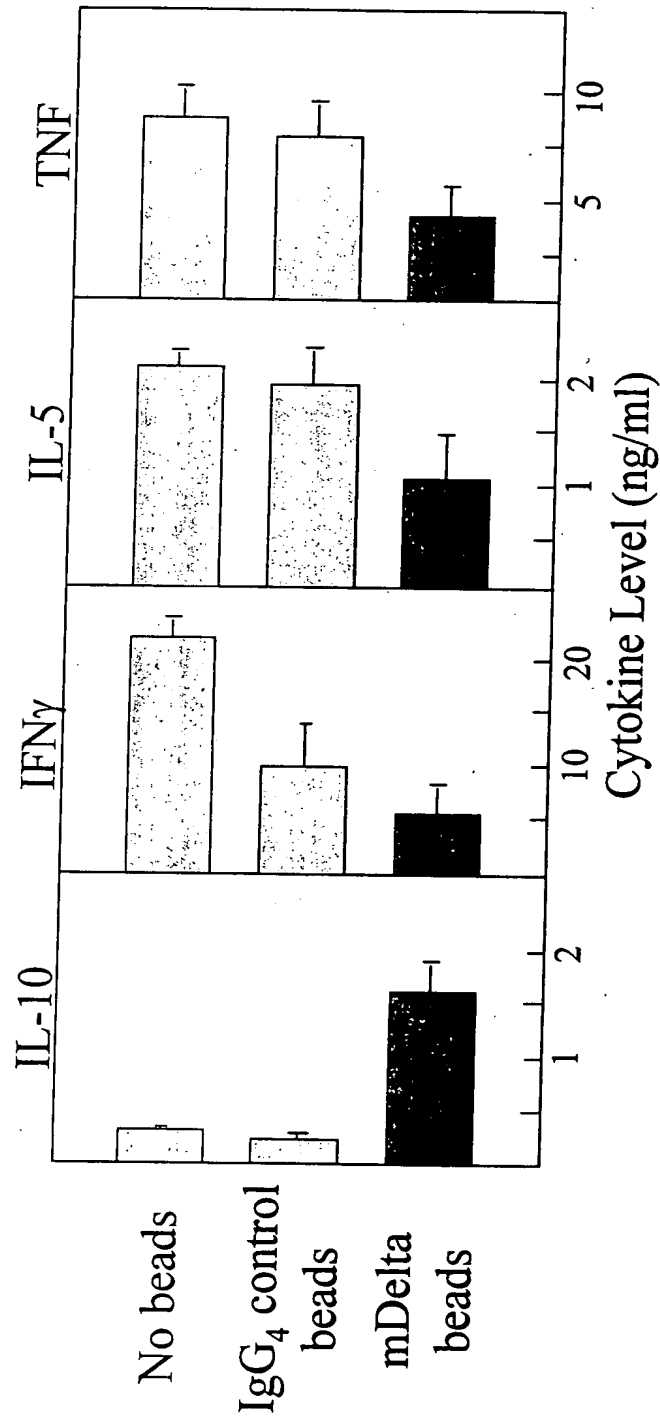


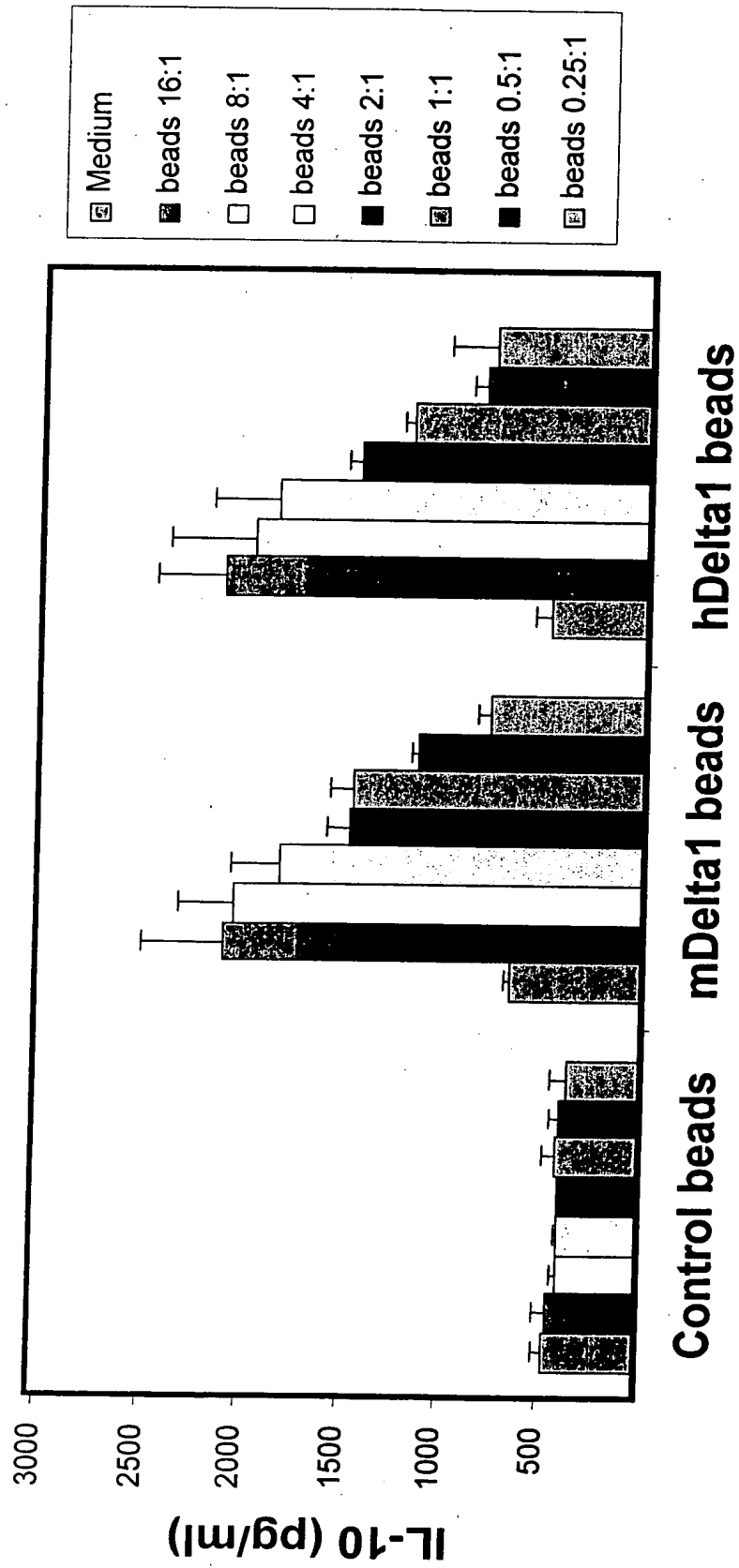
Figure 18: mDelta1-Fc Enhances IL-10 Production and decreases IFN $\gamma$ , IL-5 and TNF Production by Human CD4<sup>+</sup>T-Cells



Human CD4<sup>+</sup> T-cells stimulated with anti-CD3 + anti-CD28 with  
or without mouse Delta1-hlgG<sub>4</sub>-coated beads

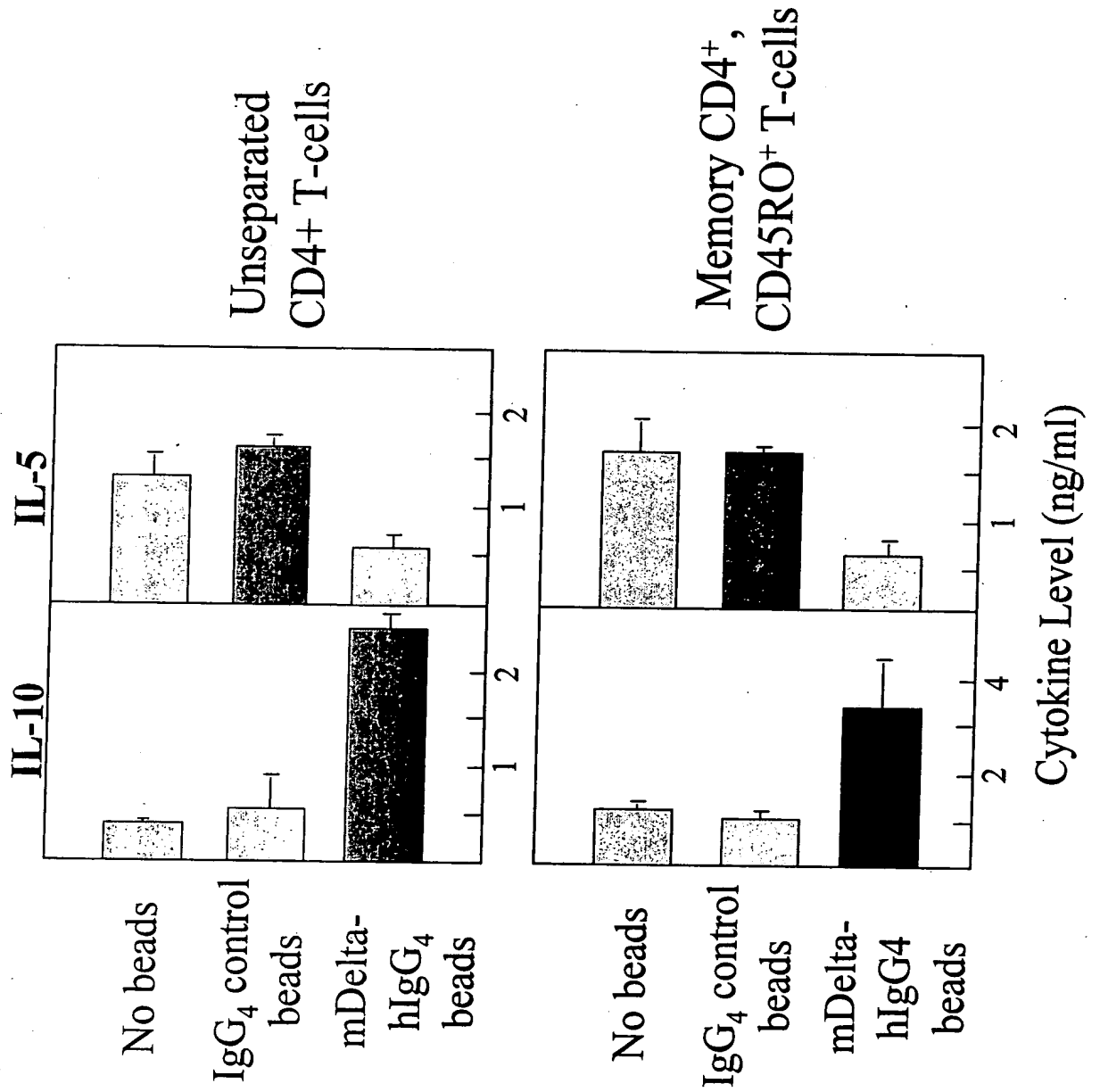


**Figure 19:** Delta1 enhances IL-10 production by human CD4<sup>+</sup> T-cells



Cells stimulated with anti-CD3/CD28 with or without Delta coated beads as shown (medium only and then bead:cell ratios 16:1, 8:1, 4:1, 2:1, 1:1, 0.5:1 and 0.25:1 from left to right in each group)

**Figure 20:** mDelta1-Fc Enhances IL-10 Production and decreases IL-5 production by Anti-CD3/CD28 Activated Human CD4<sup>+</sup> T-Cells



**Figure 21:** Delta-Fc enhances IL-10 and inhibits TNF production by murine CD4+ T-cells, even in presence of Th1 or Th2 cytokines

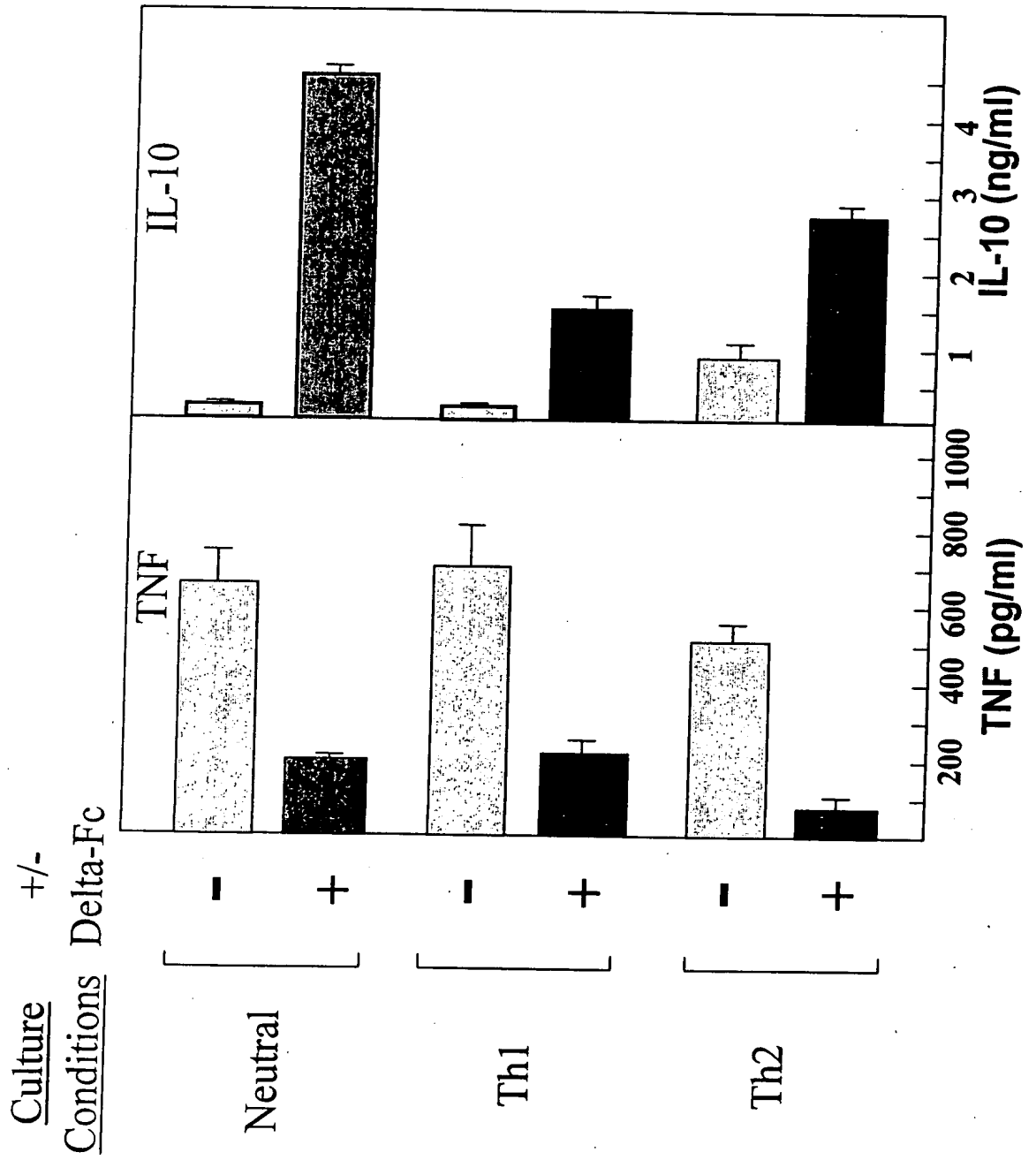


Figure 22: Micro-Array Profiling of Delta-Activated Genes in Jurkat T-Cells

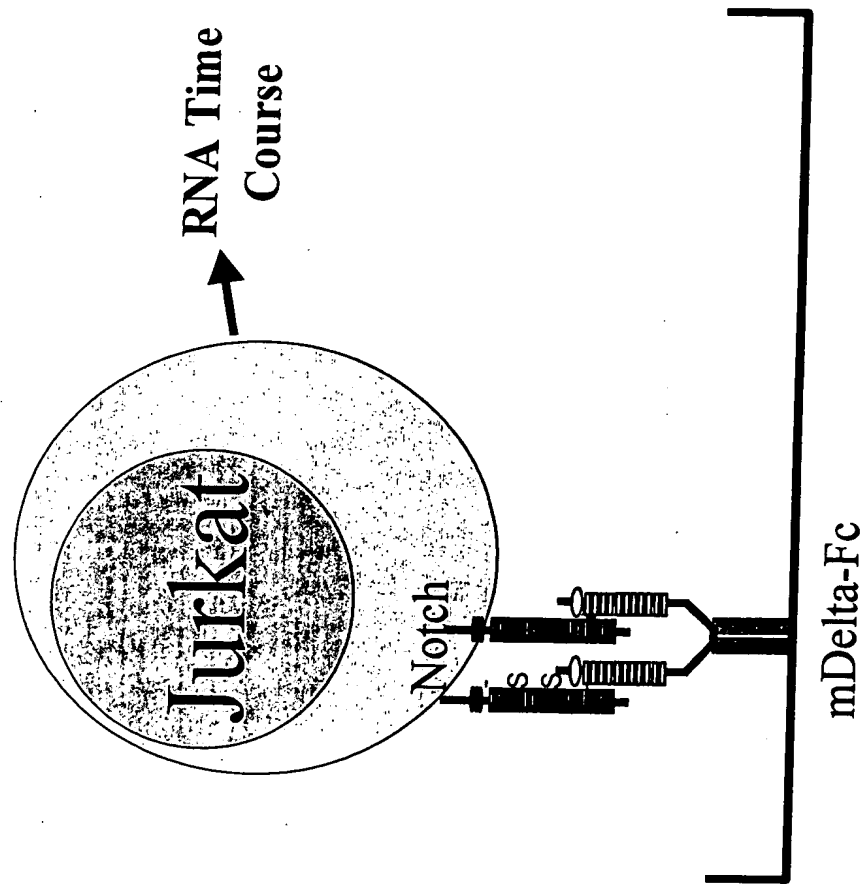


Figure 22A

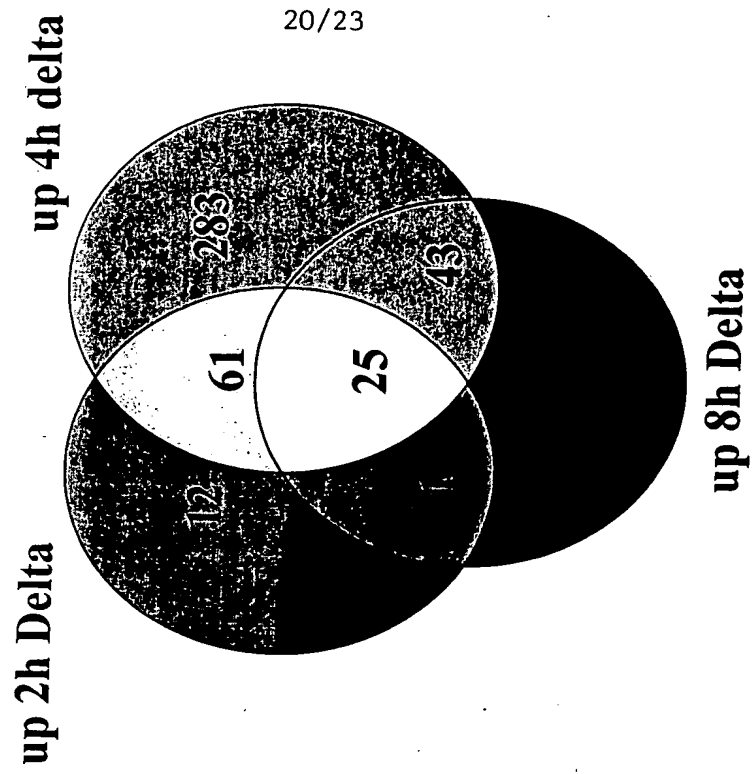


Figure 22B

**Figure 23:** Delta-Mediated Activation of Gene Expression in Jurkat T-Cells

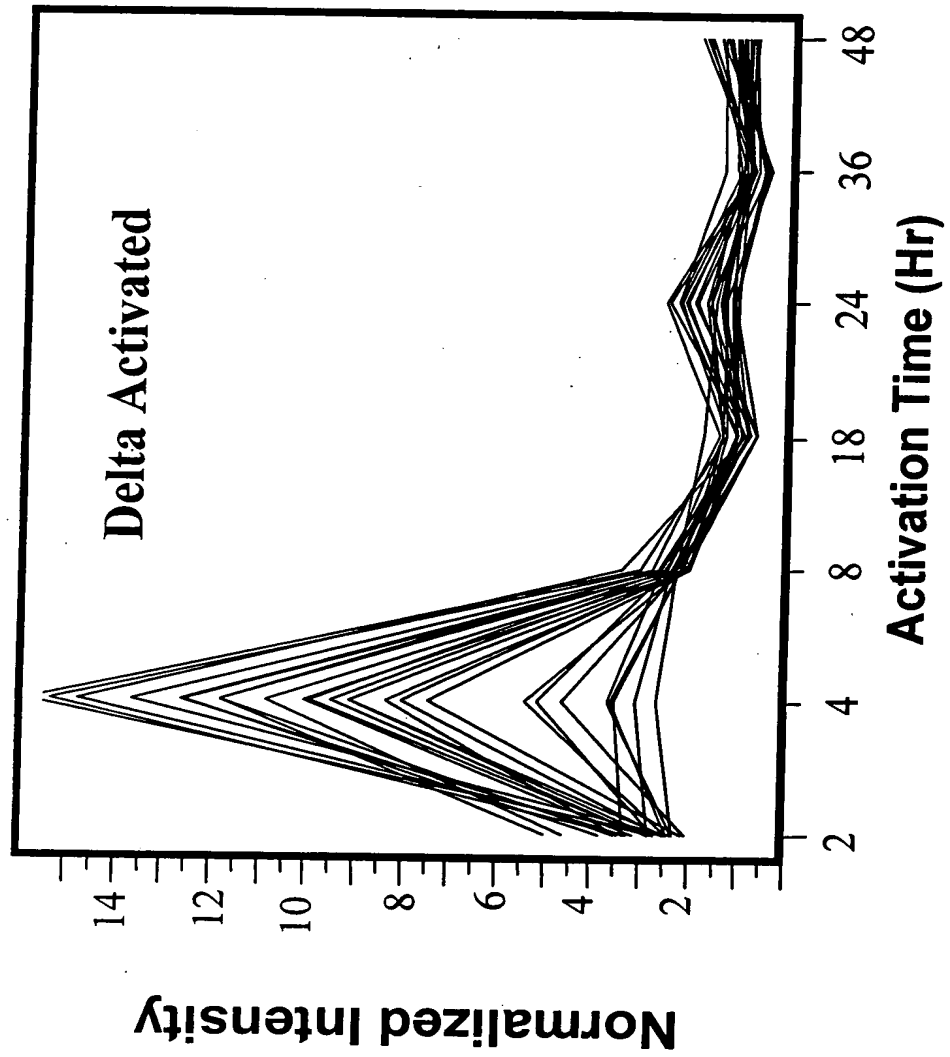


Figure 24: Micro-Array Profiling of Delta-Activated Genes in Jurkat T-Cells

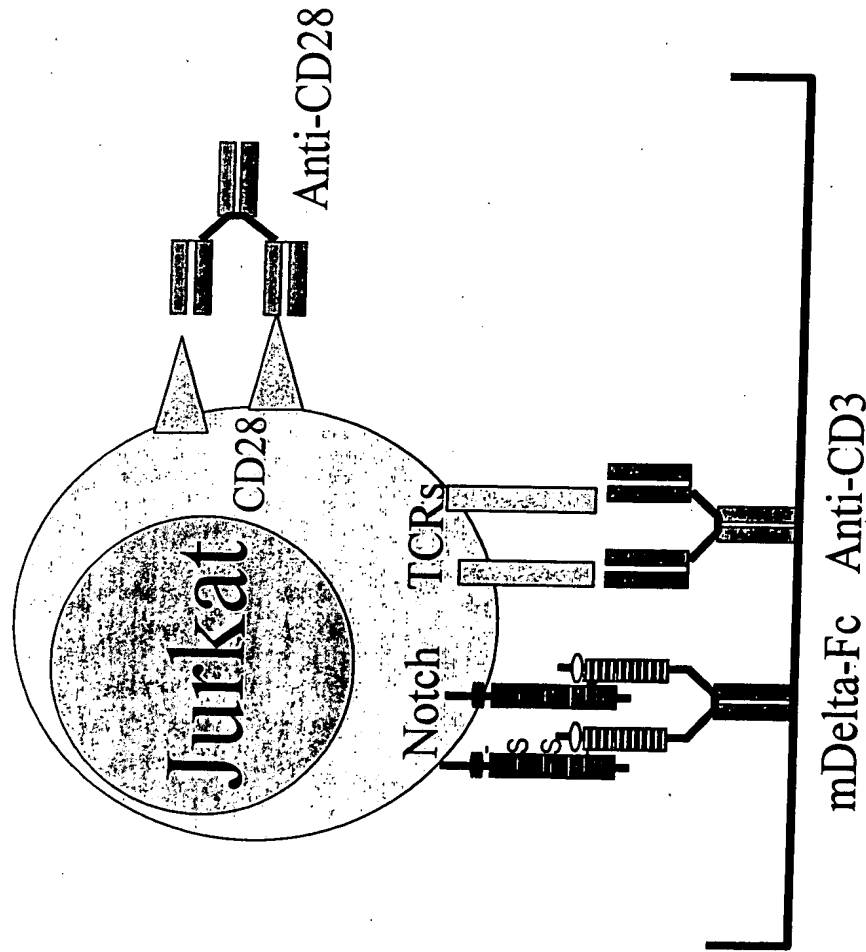


Figure 24A

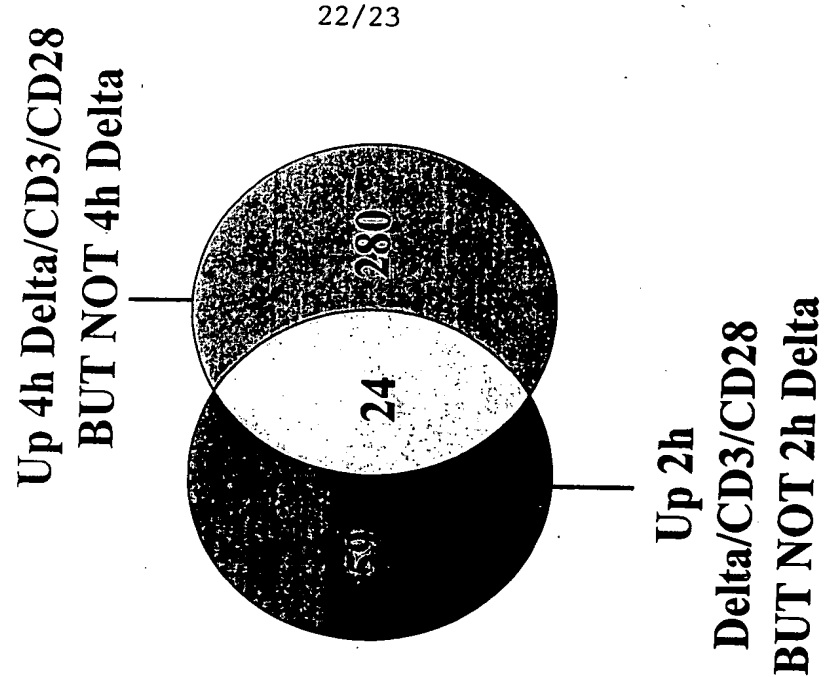


Figure 24B

Figure 25: Delta Modulation of Anti-CD3/CD28 Activation of Gene Expression in Jurkat T-Cells

